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FRONTIER

1 Advances in traction methods for endoscopic submucosal dissection: What is the best traction method and traction direction?

Nagata M

23 Gastric pentadecapeptide BPC 157 in cytoprotection to resolve major vessel occlusion disturbances, ischemia-reperfusion injury following Pringle maneuver, and Budd-Chiari syndrome

Sikiric P, Skrtic A, Gojkovic S, Krezic I, Zizek H, Lovric E, Sikiric S, Knezevic M, Strbe S, Milavic M, Kokot A, Boban Blagaic A, Seiwerth S

REVIEW

47 Transfusion-transmitted hepatitis E: What we know so far?

Cheung CKM, Wong SH, Law AWH, Law MF

76 Viral hepatitis in 2021: The challenges remaining and how we should tackle them

Dunn R, Wetten A, McPherson S, Donnelly MC

MINIREVIEWS

96 Risk of hepatocellular carcinoma after hepatitis C virus cure

Luna-Cuadros MA, Chen HW, Hanif H, Ali MJ, Khan MM, Lau DTY

108 Artificial intelligence in the diagnosis and management of colorectal cancer liver metastases

Rompianesi G, Pegoraro F, Ceresa CD, Montalti R, Troisi RI

ORIGINAL ARTICLE

Basic Study

123 Focal adhesion kinase-related non-kinase ameliorates liver fibrosis by inhibiting aerobic glycolysis *via* the FAK/Ras/c-myc/ENO1 pathway

Huang T, Li YQX, Zhou MY, Hu RH, Zou GL, Li JC, Feng S, Liu YM, Xin CQ, Zhao XK

Retrospective Cohort Study

140 Dynamics of cytokines predicts risk of hepatocellular carcinoma among chronic hepatitis C patients after viral eradication

Lu MY, Yeh ML, Huang CI, Wang SC, Tsai YS, Tsai PC, Ko YM, Lin CC, Chen KY, Wei YJ, Hsu PY, Hsu CT, Jang TY, Liu TW, Liang PC, Hsieh MY, Lin ZY, Chen SC, Huang CF, Huang JF, Dai CY, Chuang WL, Yu ML

SYSTEMATIC REVIEWS

154 Current guidelines for the management of celiac disease: A systematic review with comparative analysis

Raiteri A, Granito A, Giamperoli A, Catenaro T, Negrini G, Tovoli F

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Weekly Volume 28 Number 1 January 7, 2022

Contents

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FRONTIER

Advances in traction methods for endoscopic submucosal dissection: What is the best traction method and traction direction?

Mitsuru Nagata

ORCID number: Mitsuru Nagata 0000-0002-5697-5953.

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Mitsuru Nagata, Department of Endoscopy, Shonan Fujisawa Tokushukai Hospital, Kanagawa 251-0041, Japan

Corresponding author: Mitsuru Nagata, MD, Chief Doctor, Department of Endoscopy, Shonan Fujisawa Tokushukai Hospital, 1-5-1 Tsujidokandai, Fujisawa, Kanagawa 251-0041, Japan. mitsuru10jp@yahoo.co.jp

Abstract

Endoscopic submucosal dissection (ESD) has been developed as a treatment for superficial gastrointestinal neoplasms, which can achieve en bloc resection regardless of the lesion size. However, ESD is technically difficult because endoscopists cannot bring their hand into the gastrointestinal tract, unlike surgeons in regular surgery. It is difficult to obtain sufficient tension in the dissection plane and a good field of vision. Therefore, ESD is associated with a long procedure time and a high risk of adverse events in comparison with endoscopic mucosal resection. Traction methods have been developed to provide sufficient tension for the dissection plane and a good field of vision during the ESD procedure. However, traction direction is limited in most traction methods, resulting in insufficient effect in some cases. Although traction direction is considered important, there have been few investigations of its effect. In the first half of this review, important traction methods are discussed, including traction direction. In second half, appropriate traction methods for each organ are considered. Other important considerations for traction method, such as ability to adjust traction strength, interference between traction device and endoscope, and the need for specialized devices are also discussed.

Key Words: Endoscopic submucosal dissection; Traction method; Countertraction; Traction direction; Vertical traction

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Core Tip: Endoscopic submucosal dissection is associated with a long procedure time and adverse events (*e.g.*, perforation) due to technical difficulty—the absence of tension for the dissection plane and poor field of vision. Traction methods allow efficient dissection and a good field of vision. Although many traction methods have

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been developed, traction direction is limited in most. Each traction method has advantages and disadvantages. It is important to select an appropriate traction method to obtain proper traction direction, depending on lesion location. We discuss the characteristics of different traction methods and their effects depending on traction direction.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) allows en bloc resection, regardless of lesion size, where endoscopic mucosal resection (EMR) is considered impossible, enabling accurate pathological assessment and a lower recurrence[1]. However, ESD is still a challenging therapy due to technical difficulty, which results in long procedure time and high perforation rate[2]. Surgeons may use the nondominant hand to provide traction for the lesion while they dissect using the dominant hand. By contrast, endoscopists cannot use their nondominant hand to provide traction for the lesion during ESD because they cannot put their hand into the gastrointestinal tract—it is like cutting a steak using only a knife, with no fork. It is important to obtain traction during ESD because this enables two important effects: creating a visual field by turning over the mucosal flap, and facilitating dissection by providing tension for the dissection plane. A basic hood attached to the endoscope can be used to obtain traction. However, this is occasionally insufficient.

The clip-with-line method (Figure 1), which may be the first traction method ever used, was reported in 2002[3,4]. Many other methods have since been developed. Although the clip-with-line method is simple and low cost, its traction direction is limited to the direction in which the line is pulled. In a multicenter prospective randomized controlled trial comparing the conventional and the clip-with-line methods, the clip-with-line method did not demonstrate a reduction in procedure time for gastric ESD[5] but did for esophageal ESD[6]. These results suggest the efficacy of the traction method is different depending on traction direction, because traction direction in the clip-with-line method is limited to the direction toward the endoscope in esophageal ESD, while it changes depending on the lesion location in gastric ESD.

Unlike the clip-with-line method, several other traction methods can provide traction in any direction. These include the internal traction method, which uses an spring-and-loop with clip (S-O clip; Zeon Medical, Tokyo, Japan)[7-10] (Figure 2). We reported a single-center prospective randomized controlled trial comparing the conventional and the S-O clip-assisted methods in gastric ESD, which demonstrated that the S-O clip-assisted method reduced the median gastric ESD procedure time (29.1 min *vs* 52.6 min; $P = 0.005$)[11]. In this study, a direction vertical to the gastric wall was selected for the S-O clip-assisted method, using its multidirectional traction function.

These outcomes suggest that traction direction is the important factor for traction-assisted ESD. However, little study has been done to explore the influence of traction direction during the procedure. Each traction method has characteristics other than traction direction, and it is necessary to understand the characteristics in order to use the methods effectively. The purpose of this article is to review the characteristic of traction methods. Then follows a discussion of appropriate traction methods for each organ, based on the results of clinical trials.

BASICS OF TRACTION

Definition of traction

In published literature of ESD, the terms traction and countertraction are used interchangeably; the unclear distinction results in a potential for confusion[12]. In this

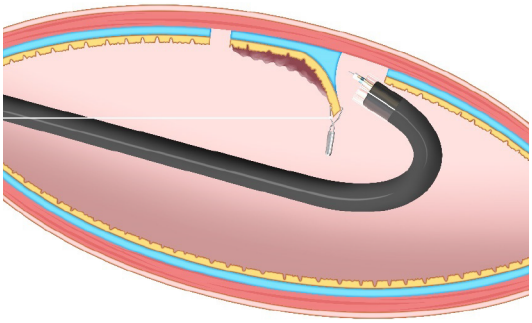


Figure 1 Clip-with-line method. This method provides traction for the lesion by pulling the line. The traction direction is limited to the direction in which the line is pulled.

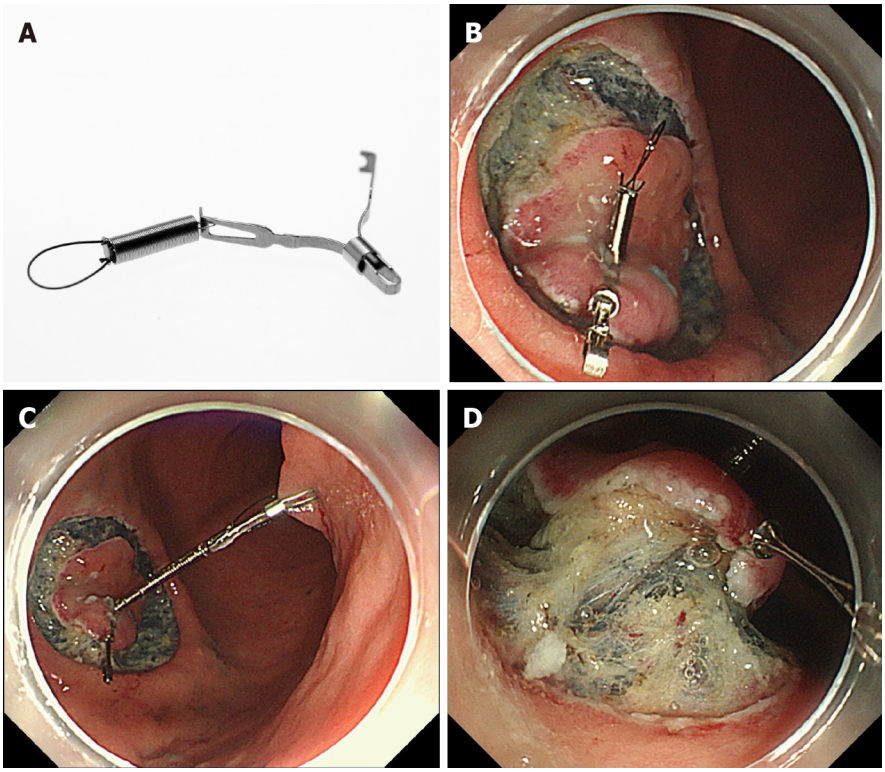


Figure 2 Internal traction method using the spring-and-loop with clip (Zeon Medical, Tokyo, Japan). A: The spring-and-loop with clip (S–O clip) has a 5-mm long spring and a 4-mm long loop at one side of the clip claws; B: The S–O clip is attached to the lesion; C: The regular clip anchors the loop of the S–O clip on the opposite side of the lesion; D: The extension of the spring provides traction on the lesion. Citation: Mitsuru Nagata. Internal traction method using a spring-and-loop with clip (S–O clip) allows countertraction in gastric endoscopic submucosal dissection. *Surg Endosc* 2020; 34(8): 3722–3733. Copyright © 2020 Mitsuru Nagata[10].

article, we do not use the term countertraction. We define traction as force acting on the target lesion.

Classification of traction direction

As a force, traction can be represented by a vector, characterized by size and direction. Traction direction can be divided into the following five categories, according to the relationship with the endoscope tip and the gastrointestinal wall: Proximal, diagonally proximal, vertical, diagonally distal, and distal (Figure 3). Of these five categories, vertical traction may be appropriate in any situation because it provides two important effects: Enabling visualization of the submucosa, by turning over the mucosa; and facilitating submucosal dissection by providing tension to submucosa (Figure 3A).

Proximal traction can provide sufficient tension to the submucosa. However, the mucosal flap falls toward the endoscope (Figure 3B). If the endoscope tip is not parallel to the gastrointestinal wall, it can be difficult to approach the submucosa, even

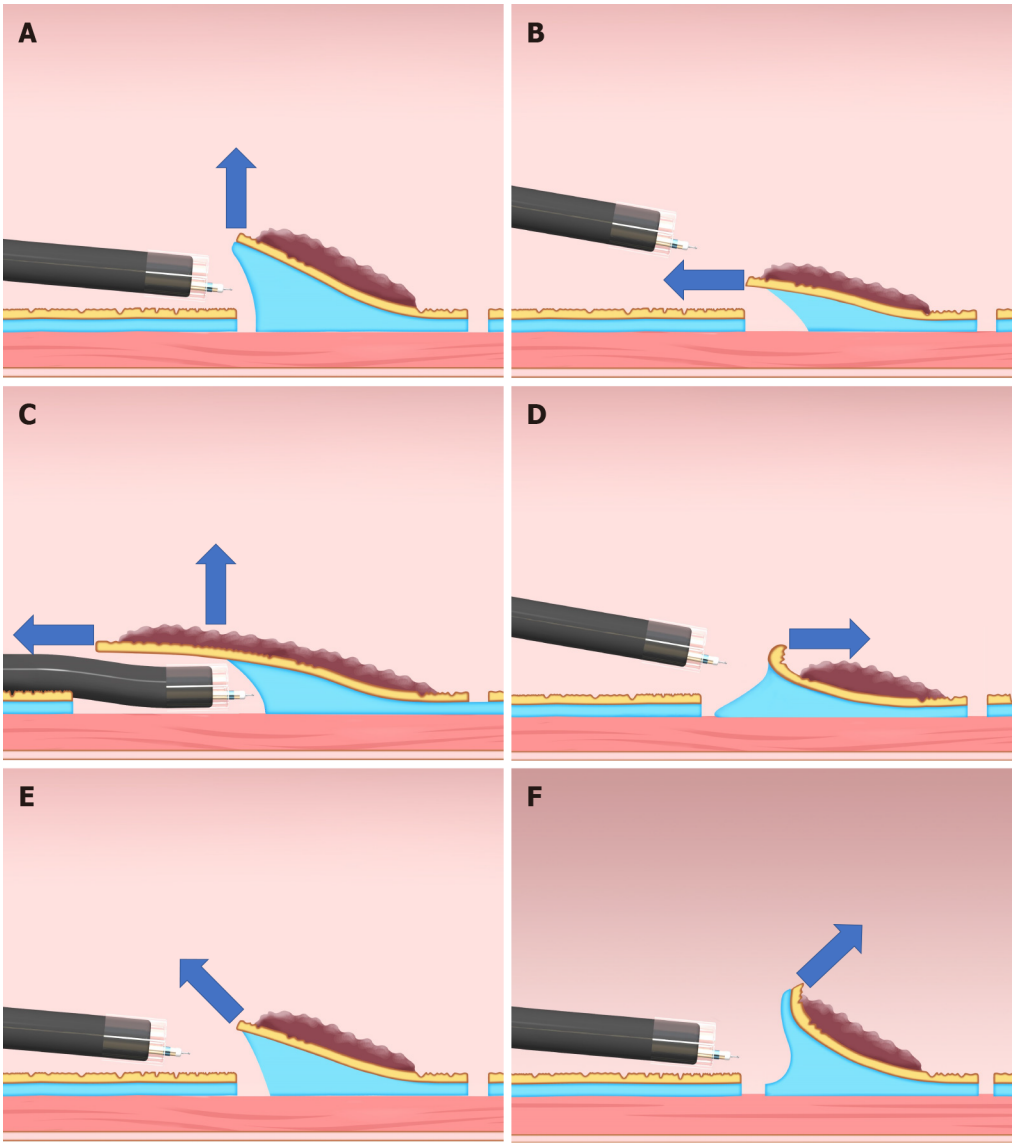


Figure 3 Classification of the traction direction. A: Vertical traction; B: Proximal traction; C: Proximal traction combined with hood traction; D: Distal traction; E: Diagonally proximal traction; F: Diagonally distal traction.

with proximal traction. If the endoscope tip is parallel to the gastrointestinal wall, it is easy to approach the submucosa, even if the mucosal flap falls down. Moreover, once the endoscope tip gets under the mucosal flap, proximal traction is combined with hood traction, resulting in diagonally proximal or vertical traction (Figure 3C). Proximal traction is suitable for situations where the endoscope tip can be placed parallel to the gastrointestinal wall, for example, in esophageal ESD.

Distal traction can cause the submucosal dissection plane to fall distally as submucosal dissection advances, resulting in submucosal thinning and subsequently, cutting the muscle layer or mucosa because of misrecognition of the layer (Figure 3D). Moreover, distal traction may decrease the effectiveness of the tension for the submucosal dissection plane, leading to inefficient dissection. Hence, distal traction may be the least useful approach for submucosal dissection in most cases.

Diagonally proximal traction (Figure 3E) and diagonally distal traction (Figure 3F) can be decomposed into horizontal and vertical vectors. The larger the horizontal component, the closer to the proximal or distal traction. The larger the vertical component, the closer to vertical traction.

MODALITY OF TRACTION

Traction can be roughly classified into hood traction, natural traction, and device-

assisted traction. Natural traction and device-assisted traction can be further subdivided.

Hood traction

A hood attached to the endoscope tip is used in ESD procedures primarily to secure the visual field. The hood also can be used to obtain traction; it can turn the mucosal flap and provide tension for the submucosa after the endoscope tip is inserted under the mucosa. The straight hood has a wide field of view but is sometimes difficult to get under the mucosa. A hood with a tapered tip may be effective in such a situation (Figure 4)[13,14]. However, in a situation where it is difficult to keep the endoscope tip under the mucosa, such as in severe submucosal fibrosis, substantial lesion movement (due to patient respiration), and vertical confrontation with the lesion, the hood alone is not effective.

Natural traction

Natural traction is defined as traction using natural power, such as gravity, mucosal tension, buoyancy, and water pressure. The advantage of natural traction is that it is easy to switch to other methods and there is no need for any special device.

Gravity: When the lesion is gravitationally upward, gravity keeps the mucosal flap open and provides tension for the submucosa (Figure 5). Changing the patient's posture to raise the lesion against gravity is the basic strategy for ESD. However, in esophageal, gastric, and duodenal ESD, the patient's posture is primarily the left lateral decubitus position, which is difficult to change. By contrast, it is easy to change the patient's posture in colorectal ESD (*e.g.*, left lateral, supine, right lateral, and prone positions). However, changing the patient's posture sometimes makes the ESD procedure complicated, for example, through poor maneuverability of the endoscope, a vertical approach to the lesion, and difficulty opening the lumen.

Mucosal tension, pocket creation method, and endoscopic submucosal tunnel dissection: When the mucosa around the lesion is incised circumferentially, the lesion loses tension from the surrounding mucosa and submucosa, making it difficult to get the endoscope tip under the mucosal flap. By leaving a part of the mucosa around the lesion, the remaining mucosa gives tension to the lesion. In conventional ESD, traction at the lesion can be maintained by using mucosal tension, as follows. A C-shaped or inverted C-shaped mucosal incision is made. Next, the submucosa under the lesion is dissected, while the remaining mucosa gives tension at the dissection plane. Finally, a circumferential mucosal incision is made and the remaining submucosa dissected.

The pocket creation method (PCM) and endoscopic submucosal tunnel dissection (ESTD) use the same principle, using mucosal tension for traction[15-19]. In PCM (Figure 6), an initial mucosal incision on the proximal side of the lesion is first performed, to make entry to the submucosa. Then, the submucosa under the lesion is dissected, followed by creation of a submucosal space. Finally, the mucosa and submucosa around the submucosal space is dissected to achieve en bloc resection. In ESTD, a mucosal incision on the distal side of the lesion is performed before creation of a submucosal space is completed, unlike the PCM procedure.

PCM and ESTD procedures have similar advantages, as follows. The endoscope inside the submucosal space provides tension for the dissection plane. The endoscope tip can take a parallel approach to the muscle layer. The submucosal space holds the endoscope, which achieves stabilization of the endoscope. Thus, PCM and ESTD may be particularly suitable for lesions that are located where maneuverability of the endoscope is poor. Moreover, minimal mucosal incision until completion of submucosal dissection may prevent leakage of the injected solution.

Buoyancy and water pressure: We have reported the usefulness of underwater ESD for buoyancy, easier use of water pressure from an endoscope that has a water supply function, clear visual field, and heat sink effect[20,21]. Buoyancy and water pressure can provide a traction function, where buoyancy acts opposite to gravitational pull.

In conventional ESD, when the lesion is located at the gravitationally lower side, the opening of the mucosal flap is obstructed by gravity. Moreover, the lesion is half-way submerged due to gravity, while the boundary between gas and liquid obstructs the visual field (Figure 7A and B). By switching from conventional ESD to underwater ESD, buoyancy aids opening of the mucosal flap and provides tension for the submucosa, while the visual field is unaffected by a gas-liquid boundary (Figure 7C and D). Water pressure from the endoscope (using its water supply function) also assists opening the mucosal flap. Whereas water pressure can temporarily deteriorate

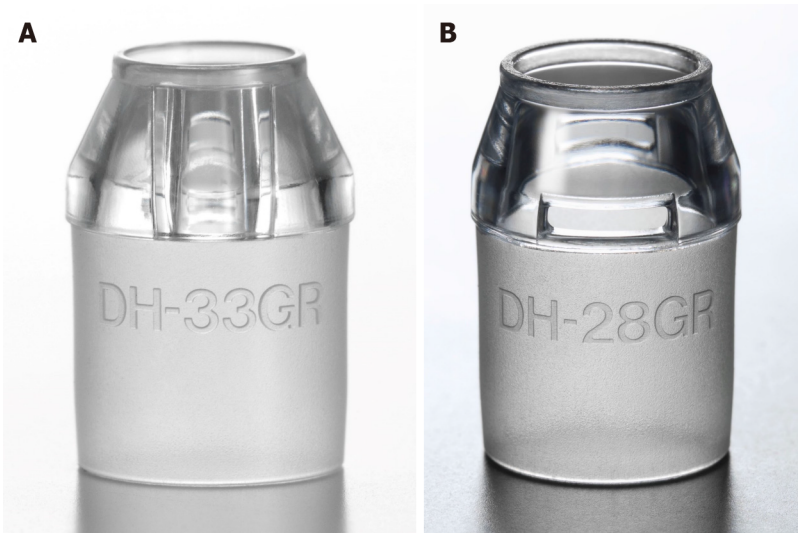


Figure 4 Small-caliber tip transparent hood (Fujifilm, Tokyo, Japan). A: DH-33GR [small-caliber tip transparent (ST) hood] with 7-mm tip opening diameter and 7-mm tip protruding length; B: DH-28GR (short ST hood) with 8-mm tip opening diameter and 7-mm tip protruding length.

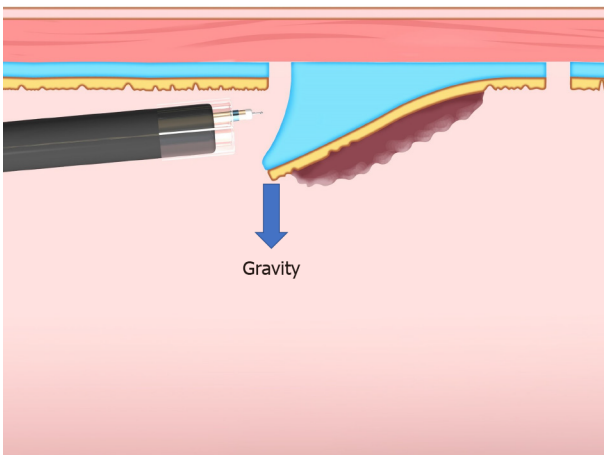


Figure 5 Gravity provides favorable traction when the lesion is located at the upper side of the gravitational force.

the visual field, due to splashing under gas insufflated conditions, it can be used without splashing in underwater conditions, allowing seamless submucosal dissection.

Saline solution is preferable for underwater ESD due to saline solution's higher specific gravity, compared with water, which provides a greater flotation effect.

Device-assisted traction method

A traction method using a device other than a hood can be defined as device-assisted. Device-assisted traction method implies traction in the narrow sense. They are broadly classified into external, internal, and other methods; each of these is further sub-classified (Table 1).

External traction methods: External traction can be defined as a method where the traction device acts from outside the body. Representative external traction method includes the clip-with-line method, pulley method, sheath traction method, external forceps method, double scope method, and magnetic anchor method.

Clip-with-line method: The clip-with-line method was reported by Oyama *et al*[3,4] in 2002 (Figure 1). It is performed as follows. After circumferential mucosal incision, the endoscope is withdrawn. The clip applicator is deployed into the accessory channel of the endoscope. The clip-with-line (a clip with a line tied to its arm) is attached to the clip applicator. The endoscope is inserted, then the clip-with-line is attached to the edge of the lesion. Using this procedure, the line comes out of the body without passing through the accessory channel of the endoscope, while pulling the line provides traction at the lesion.

Table 1 Classification of device-assisted traction method

	Traction direction	Control of traction force	Withdrawal of the endoscope	Recommended lesion location
External traction methods				
Clip-with-line method	One	Strengthen	Required	Esophagus, Greater curvature of the upper and middle third of the stomach, Colorectum
Pulley method	Any	Strengthen	Required	unclear because of fewer reports
Sheath traction method				
Clip-and-snare method	Two	Strengthen and weaken	Required	Stomach, Rectum
Endo Trac	Two	Strengthen and weaken	Required	Stomach, Rectum
External forceps method	Two	Strengthen and weaken	Required	Esophagus; Stomach except for cardia, lesser curvature or posterior wall of the upper gastric body; Rectum
Double scope method	Any	Strengthen and weaken	None	Stomach
Magnetic anchor method	Any	Strengthen and weaken	Required	Stomach, Colorectum
Internal traction method				
S–O clip	Any	Strengthen and weaken	None	Stomach, Colorectum
Ring thread	Any	Strengthen and weaken	None	Colorectum
Multiloop	Any	Strengthen and weaken	None	Colorectum
Double clip and rubber band	Any	Strengthen and weaken	None	Colorectum
Others				

The advantages of this method are its simplicity, low cost, and no requirement for a special device. The disadvantage is that traction direction is limited to the direction in which the line is pulled; therefore, submucosal dissection may be difficult, depending on traction direction. Although increasing traction force is possible, by pulling the line, it is difficult to weaken traction force. Moreover, friction between the endoscope and the line in the narrow space generates interference, which sometimes causes strong traction resulting in slip-off of the clip. In fact, the slip-off rate is reported to be 16.4% in esophageal ESD[6] and 13.2% in gastric ESD[5].

Pulley method: The pulley method is a modified clip-with-line method. By anchoring the line to the gastrointestinal wall, the direction of traction can be controlled in any direction (Figure 8). The pulley method can be classified into two types according to the pulley system used: Clip pulley[22] or suture pulley[23,24]. There are only a few reports on the pulley method, and its effectiveness needs to be verified.

Sheath traction method: In the sheath traction method, the line part of the clip-with-line method is replaced with a sheath. Since the sheath is harder than the line, it can provide not only pulling force but also pushing force to the lesion, thus allowing two traction directions (Figure 9). Sheath traction method includes the clip-and-snare method[25-29] and the Endo Trac[30,31] (TOP, Tokyo, Japan) (Figure 10A).

The clip-and-snare method requires only a polypectomy snare and a clip; therefore, this method may be performed anywhere. In the conventional clip-and-snare method, the snare is grasped with forceps and delivered to the lesion[25]. However, this procedure is sometimes difficult. The prelooping technique was developed to improve the delivery of the snare[26-29]. The prelooping technique for the clip-and-snare method is performed as follows. After circumferential mucosal incision, the endoscope is withdrawn to preloop the snare on the tip of the endoscope. Then, the endoscope is inserted to the lesion along with the snare sheath. The clip is attached to the edge of

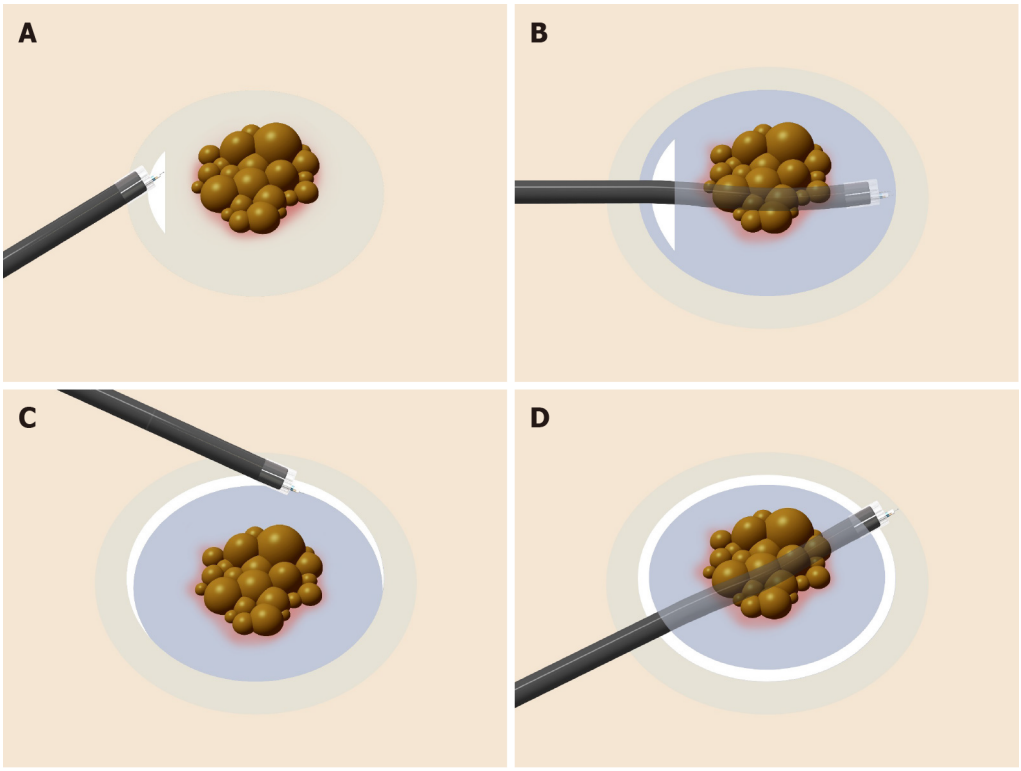


Figure 6 Procedure for the pocket creation method of endoscopic submucosal dissection. A: A minimal initial mucosal incision (approximately 20-mm in width) is made for entry into the submucosa; B: Submucosal dissection is performed, followed by creation of a submucosal pocket under the lesion; C: Additional mucosal incision of the gravitational lower side of the submucosal pocket; D: Opening around the submucosal pocket, then, en bloc resection is achieved.

the lesion. Then, the snare is loosened so that it can slide over the clip applicator toward the clip. Finally, the snare holds the clip, and the clip applicator is withdrawn.

Endo Trac is a product developed for the sheath traction method. Whereas interference between the sheath and the tip of the endoscope sometimes makes access to the submucosa difficult when the clip-and-snare method is used, the Endo Trac has a structure that can release the sheath from the lesion, to avoid interference between the sheath and the endoscope tip (Figure 10B).

The clip-and-snare method and the Endo Trac require withdrawal and reinsertion of the endoscope to set the traction system. Therefore, these methods are not suitable for colonic lesions, in which insertion of the endoscope is difficult and time-consuming. Moreover, interference between the sheath and the endoscope, due to friction, is possibly greater than with the clip-with-line method, due to the sheath being thicker than the line. In fact, it has been reported that even with a thin sheath (with a maximum diameter of 1.8 mm), interference with the endoscope can occur to some extent; the operator needs to move the endoscope carefully to avoid detachment of the snare from the clipped lesion[29].

External forceps method: The external forceps method is performed as follows[32-36]. After circumferential mucosal incision, the endoscope is withdrawn. The endoscope is reinserted with external forceps that are grasped by second forceps inserted through the accessory channel of the endoscope. External forceps grasp the edge of the lesion (Figure 11A) while the second forceps are withdrawn. The external forceps provide traction to two directions by pulling or pushing the lesion (Figure 11B). This method allows changing the traction point, by releasing and re-grasping the lesion.

However, this method has some disadvantages. It is difficult to deliver the external forceps, depending on lesion location, such as the cardia, lesser curvature or posterior wall of the upper gastric body, duodenum, and colon. Interference between the endoscope and the external forceps may be relatively strong compared with that of the clip-with-line and the sheath traction methods, because the forceps is thicker than the line and the sheath. Great care should be taken regarding potential damage to mucosa grasped by the external forceps, because of the strong traction.

Double scope method: The double scope method is performed by two experienced endoscopists with main and second endoscopes[37,38]. The second endoscope is inserted alongside the main endoscope. Then, the second endoscope deploys the forceps, through the accessory channel, and grasps the lesion to provide traction. A

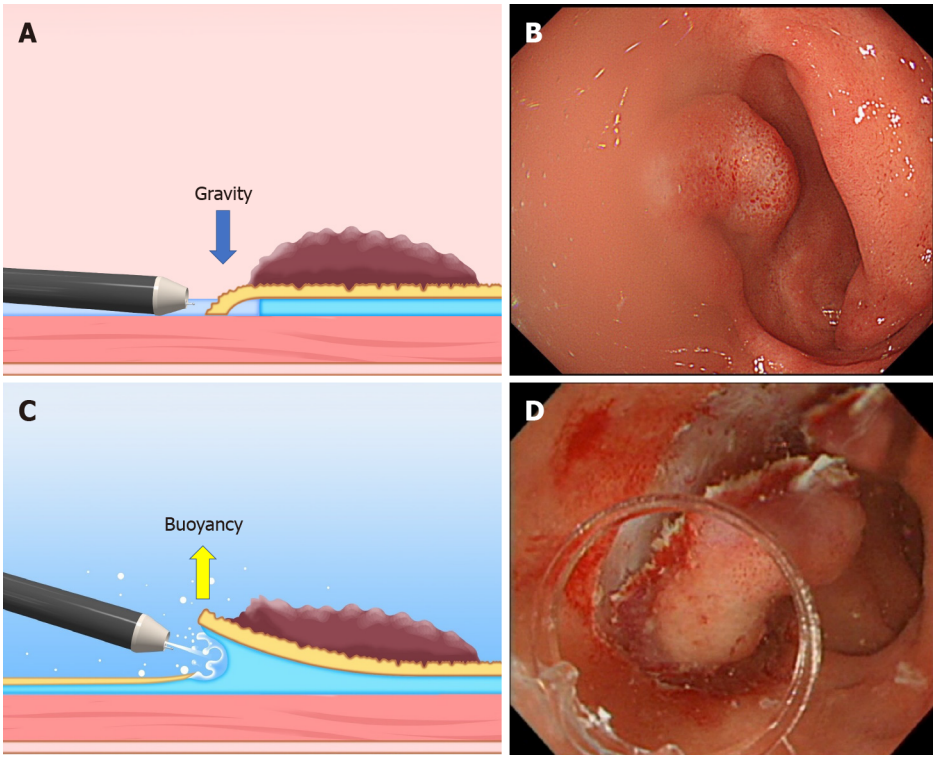


Figure 7 The difference between the conventional endoscopic submucosal dissection and the underwater endoscopic submucosal dissection. A and B: The conventional endoscopic submucosal dissection for the lesion is located at the gravitational lower side. Gravity obstructs the opening of the mucosal flap. Incomplete submersion deteriorates the visual field; C and D: The underwater condition aids the opening of the mucosal flap by buoyancy. Water pressure from the endoscope (using its water supply function) also assists in opening the mucosal flap. Complete submersion improves the visual field. Citation: Mitsuru Nagata. Underwater endoscopic submucosal dissection in saline solution using a bent-type knife for duodenal tumor. *VideoGIE* 2018; 3(12): 375–377. Copyright © 2018 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[21].

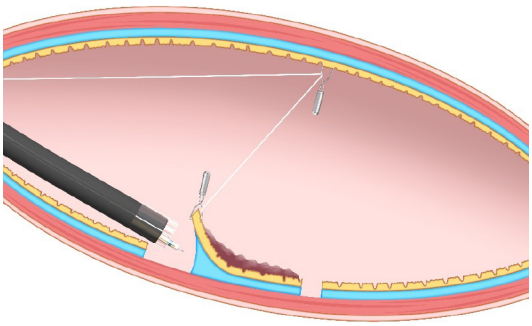


Figure 8 Pulley method. This method is the modified clip-with-line method, which can provide traction in any direction depending on the pulley site.

thin endoscope is recommended for the second endoscope, to avoid interference with the main endoscope.

This method has the great advantage that traction direction can be easily controlled by the second endoscope. Although the indication may be limited, as this method requires two experienced endoscopists and two endoscope systems, it may be a useful option for difficult cases, such as gastric cancers with ulcer scar[39]. This method has been reported to be useful in the treatment of superficial pharyngeal cancers[40] and gastric submucosal tumors[41].

Magnetic anchor method: The magnetic anchor method, as initially reported, used a large external electromagnet to provide traction, by moving an internal magnet attached to the lesion[42,43]. However, it was necessary to miniaturize the external electromagnet in clinical practice. Recently, use of neodymium rare earth magnets has allowed the external electromagnet to be minimized[44,45]; the feasibility of this method in clinical practice has been demonstrated in a prospective trial[46]. Although this method requires a special magnetic device and is not yet widespread, it is a promising method for the future due to the great advantage that it can provide

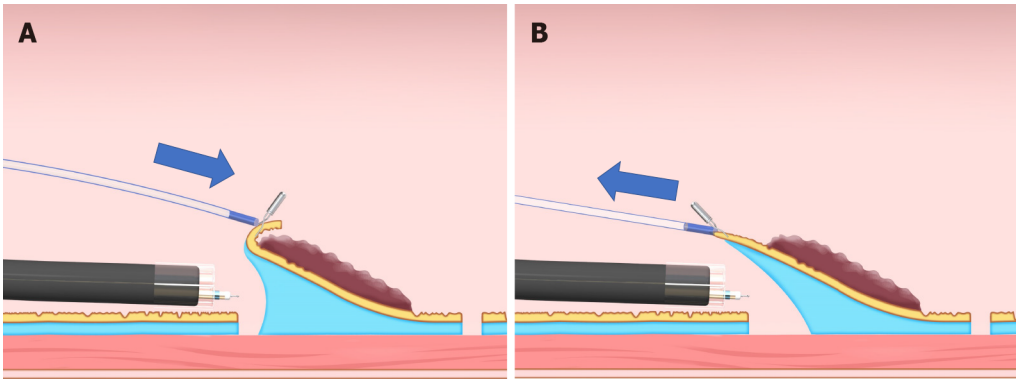


Figure 9 Sheath traction method. This method has two traction directions by pushing or pulling the sheath. A: Pushing the sheath; B: Pulling the sheath.

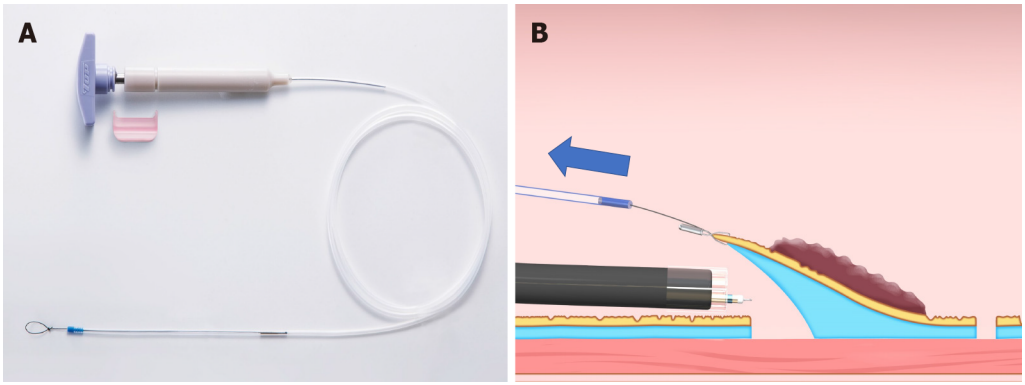


Figure 10 Endo Trac (TOP, Tokyo, Japan). A: This device can be used for the sheath traction method; B: This device has a structure that can release the sheath from the lesion to avoid interference between the sheath and the endoscope tip.

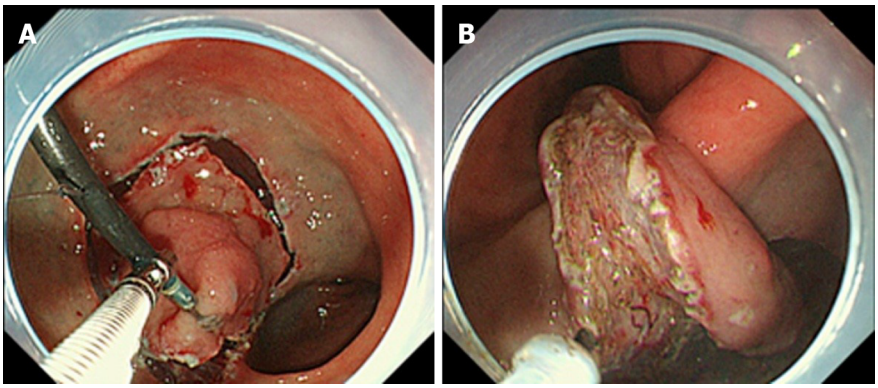


Figure 11 Endoscopic submucosal dissection using external forceps. A: External grasping forceps was anchored at the distal margin of the lesion in the lesser curvature of the antrum under the control of the endoscope and a second grasping forceps; B: With gentle oral traction applied with the external grasping forceps, the submucosal layer was dissected in retroversion from the aboral side. Citation: Imaeda H, Hosoe N, Kashiwagi K, Ohmori T, Yahagi N, Kanai T, Ogata H. Advanced endoscopic submucosal dissection with traction. *World J Gastrointest Endosc* 2014; 6(7): 286-295. Copyright © 2014 Baishideng Publishing Group Inc. Published by Baishideng Publishing Group Inc[35].

traction in any direction and control traction force. It should be noted that patients with a cardiac pacemaker or implantable cardioverter-defibrillator are not indicated for this method.

Internal traction methods: An internal traction method can be defined as a method in which the traction device acts only inside the gastrointestinal tract. Devices for internal traction include the S-O clip[7,8] (Zeon Medical, Tokyo, Japan) (Figure 2), ring thread [47], multiloop[48], double clip and rubber band[49], and clip band[50]. The principle for generating traction is the same in these devices, as follows. First, the clip with the

specific mechanism (*e.g.*, spring, thread, and band) for generating the traction is attached to the lesion (Figure 12A). Second, the regular clip anchors the tip of the mechanism to the gastrointestinal wall (Figure 12B). Finally, extension of the mechanism provides traction to the lesion (Figure 12C).

In these devices, the traction direction can be controlled in any direction by the anchor site. Traction force can also be controlled to some extent by inflating or collapsing the lumen. These devices are useful especially for colorectal ESD, as withdrawal of the endoscope is not required. The disadvantage is that a certain distance between the anchor clip and the clip attached to the lesion is required to generate the traction force. Therefore, these devices are usually utilized for gastric ESD [9-11] or colorectal ESD[47-50].

These devices are designed primarily for use with the forward endoscopic position, because there is a possibility that, in the retroflexed position, the endoscope may stretch the traction device, resulting in laceration of the mucosal flap or slip-off or breakage of the traction device. In gastric ESD, the retroflexed endoscopic position is as common as the forward position, due to the large lumen, unlike colorectal ESD. Therefore, we developed a modified method for attaching the S–O clip, to avoid stretching of the spring by the endoscope[9-11] (Figure 13). Although there are several devices for internal traction, the S–O clip may be the most appropriate in gastric ESD, as the S–O clip has a spring with higher elasticity than a thread or band. The elasticity of the spring can be easily adjusted for a large lumen, preventing laceration of the mucosal flap, slip-off, or breakage of the traction device. The S–O clip is sold only in Japan currently. However, it will be sold in future in Asian countries under the brand name "Countertraction CLIP".

The management of the anchor clip after traction is not standardized. Conventionally, the traction mechanism of the device (*e.g.*, thread, band) is cut to detach the resected specimen, while the anchor clip remains on the gastrointestinal wall. In colorectal ESD, the anchor clip may naturally drop by vermiculation. In contrast, vermiculation of the stomach is poor, except in the pars pylorica; there is a possibility of a permanent residual of the anchor clip after gastric ESD[51]. Therefore, we usually detach the anchor clip with forceps. So far, we have not experienced any adverse events from detaching the anchor clip (*e.g.*, perforation, post-ESD bleeding from the anchor site), probably because of the thicker stomach wall, compared with other organ of the gastrointestinal tract[10,11]. The safety of this management method for the anchor clip should be assessed in many gastric cases.

Others

Pocket creation method with traction device: The combination of the PCM and traction device (TD) for internal traction has been reported to facilitate better mucosal flap formation and opening of the submucosal pocket, compared with conventional PCM[52]. A retrospective study demonstrated that the median dissection speed in PCM with TD was significantly greater than in conventional ESD with TD (16.6 mm^2/min *vs* 12.2 mm^2/min; P = 0.003)[53]. Additional studies are needed to confirm whether TD has an additional effect in PCM, by comparing PCM alone against PCM with TD.

Clip flap method: The clip flap method has been reported as using a clip attached to the edge of the lesion to substitute for the mucosal flap until it is made (Figure 14)[54-56]. By using the clip flap method together with clip-based traction (*e.g.*, clip-with-line), the procedure of getting under the mucosal flap can be facilitated, especially when proximal traction makes the mucosal flap fall down (Figure 3B). Although a randomized controlled trial comparing the conventional method and clip flap method in gastric ESD demonstrated that the clip flap method had no advantage in efficacy and safety[57], this method may be effective when it is used along with other clip-based traction methods.

TRACTION METHODS ASSOCIATED WITH LESION SITE

Esophageal ESD

Representative traction methods that are reported to be effective in esophageal ESD include the clip-with-line method and ESTD. A multicenter randomized controlled trial demonstrated that the median ESD procedure time was significantly shorter with the clip-with-line method (n = 116) than with the conventional method (n = 117) (44.5

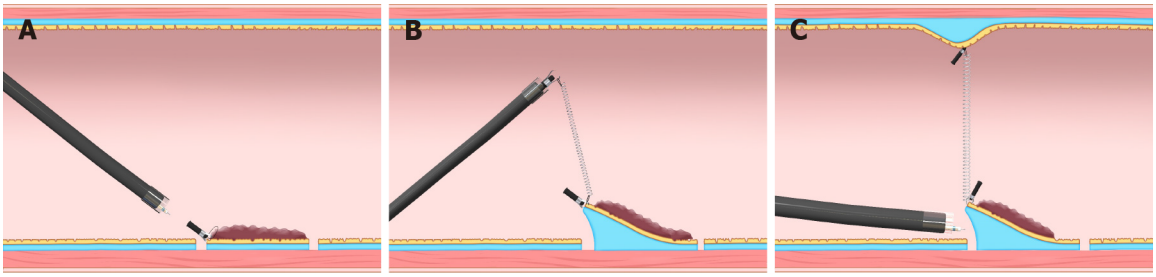


Figure 12 Internal traction method using the spring-and-loop with clip (Zeon Medical, Tokyo, Japan). A: The spring-and-loop with clip (S–O clip) is attached to the lesion; B: The regular clip anchors the loop part of the S–O clip on the gastrointestinal wall; C: The extension of the spring provides traction on the lesion. The traction direction can be controlled by the anchor site. Citation: Nagata M, Fujikawa T, Munakata H. Comparing a conventional and a spring-and-loop with clip traction method of endoscopic submucosal dissection for superficial gastric neoplasms: a randomized controlled trial (with videos). *Gastrointest Endosc* 2021; 93(5): 1097-1109. Copyright © 2021 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[11].

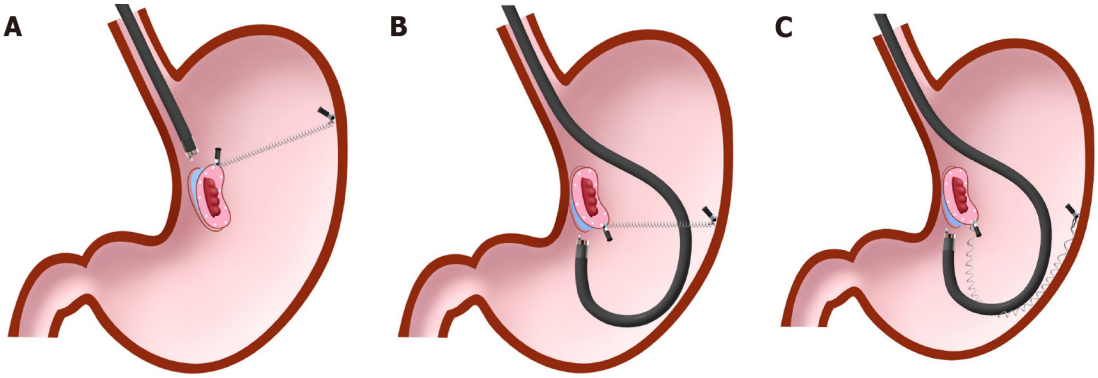


Figure 13 Spring-and-loop with clip-assisted gastric endoscopic submucosal dissection. A: Forward endoscopic position; B: Retroflexed endoscopic position; C: The endoscope has the possibility to stretch the spring, resulting in a loss of spring elasticity in the retroflexed endoscopic position. In this situation, the modified attachment method that we described in detail in the previous papers[9-11] is required to avoid this problem. Citation: Nagata M, Fujikawa T, Munakata H. Comparing a conventional and a spring-and-loop with clip traction method of endoscopic submucosal dissection for superficial gastric neoplasms: a randomized controlled trial (with videos). *Gastrointest Endosc* 2021; 93(5): 1097-1109. Copyright © 2021 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[11].

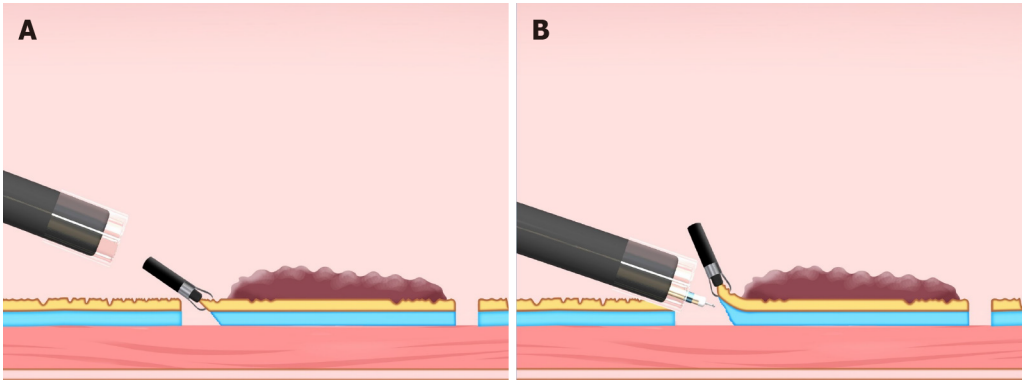


Figure 14 Clip flap method. A: The clip is attached to the edge of the lesion; B: The clip can be used as a substitute for the mucosal flap.

min *vs* 60.5 min; $P < 0.001$)[6]. Although traction using the clip-with-line method in esophageal ESD is limited to proximal traction, because the forward endoscopic position is predominantly used, due to the narrow cylindrical esophageal lumen, proximal traction may be effective, because the endoscope tip can approach parallel to the esophageal wall and can easily access the submucosa without vertical traction. After getting under the mucosal flap, hood traction and proximal traction using the clip-with-line method are combined, providing diagonally proximal or vertical traction to the submucosa (Figure 3C). Remarkably, the conventional method was changed to the clip-with-line method in six patients (5.2%) because of technical

difficulties. Moreover, five patients (4.3%) experienced perforation under the conventional method, whereas one patient (0.9%) could not complete the ESD procedure because of perforation. Conversely, no perforations were observed in the clip-with-line method.

In a multicenter randomized controlled trial, the clip slip-off rate was reported as 16.4%[6]. If clip slip-off occurs, there is a possibility that histopathological evaluation for the margin is made difficult due to damage to the specimen. Moreover, clip slip-off requires reattaching the clip-with-line, which is time-consuming. Interference between the endoscope and the line due to friction causes slip-off. Therefore, once the clip-with-line is attached to the lesion, unnecessary movement and withdrawal of the endoscope should be avoided.

A propensity score matching analysis[58] showed that ESTD had a shorter median ESD procedure time (38.0 min *vs* 48.0 min; $P = 0.006$) and lower muscle injury rate (28.9% *vs* 52.6%; $P = 0.036$) compared with conventional ESD. Furthermore, a meta-analysis including 17 studies[59] showed that ESTD had significantly higher en bloc resection rate, shorter ESD procedure time, and lower muscle injury rate. In ESTD, the endoscope tip is held by the submucosal tunnel, which allows stabilization of the endoscope and a parallel approach to the muscle layer. The endoscope tip inside the submucosal tunnel pushes up the lesion, providing sufficient tension at the dissection plane. These advantages of the ESTD may provide a shorter ESD procedure time and a lower muscle injury rate.

In conclusion, many promising results have been reported for the clip-with-line method and ESTD. At present, it may be better to select either of these two methods. Most studies on traction method for esophageal ESD have been reported from Asia, mainly targeting squamous cell carcinoma. There are not many reports of traction-assisted ESD for Barrett's esophageal adenocarcinoma, located around the esophagogastric junction; future studies should focus on this issue.

Gastric ESD

As the stomach lumen is large, both the forward and retroflexed endoscopic positions are common, unlike in esophageal, duodenal, and colonic ESD. Therefore, it is desirable that a traction method for gastric ESD is easy to utilize in both forward and retroflexed endoscopic positions. The popular traction methods for gastric ESD include clip-with-line, internal traction, sheath traction, and ESTD.

The clip-with-line method may be the first traction method for gastric ESD, and was reported in 2002[3,4]. However, a multicenter randomized controlled trial[5] comparing the conventional ESD ($n = 316$) and the clip-with-line method ($n = 319$) failed to show a reduction in the mean procedure time for gastric ESD in the total population (conventional ESD, 60.7 min *vs* clip-with-line method, 58.1 min; $P = 0.45$). Since traction by the clip-with-line method in gastric ESD is limited to the cardia, the direction of traction varies depending on the lesion location. In the retroflexed endoscopic position, the traction is likely to be distal, especially for lesions located at the lesser couverture side of the gastric body (Figure 15A). Distal traction may cause the submucosal dissection plane to fall distally, making the procedure difficult and prolonging the procedure time in some cases. In the forward endoscopic position, the traction may be proximal or diagonally proximal (Figure 15B). If the endoscope tip cannot be parallel to the gastric wall, proximal traction may cause the mucosal flap to fall proximally, making it difficult to approach the submucosal layer. In contrast, a subgroup analysis based on lesion location demonstrated that the mean ESD procedure time for lesions located at the greater curvature of the upper and middle third of the stomach was significantly shorter in the clip-with-line method (104.1 min *vs* 57.2 min; $P = 0.01$). From an anatomical point of view, these results seem reasonable, because it is difficult for the clip-with-line method to provide vertical traction unless the lesion is located at the greater curvature (Figure 15C). In a subgroup analysis based on operator experience, the mean ESD procedure time was not significantly different between the conventional and clip-with-line methods in an expert group (58.0 min *vs* 58.0 min; $P = 1.00$). Conversely, the mean ESD procedure time in a trainee group tended to be better in the clip-with-line method (68.9 min *vs* 58.3 min; $P = 0.13$). However, the lesions managed by trainees were primarily easy cases; therefore, a simple comparison may be inaccurate. Nonetheless, the analysis result suggests that the benefit from clip-with-line method differs depending on the operator experience.

S–O clip-assisted ESD is classified as internal traction, and can provide traction in any direction. Since the use of the S–O clip in gastric ESD has a potential for the endoscope in the retroflexed position to stretch the spring, we have developed a modified attachment method for the S–O clip, to avoid interference between the endoscope and spring part of the clip[9,10]. We reported a single-center randomized

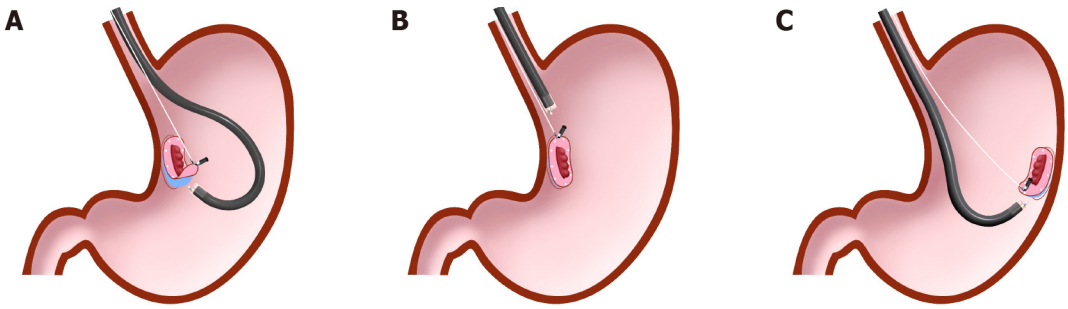


Figure 15 Difference in traction direction depending on the lesion location in the clip-with-line method. A: Distal traction; B: Proximal traction; C: Vertical traction.

controlled trial comparing conventional (n = 40) and S–O clip-assisted ESD (n = 40), which showed that the median ESD procedure time was significantly shorter in S–O clip-assisted ESD than in conventional ESD (29.1 min *vs* 52.6 min; P = 0.005)[11]. According to the subgroup analysis comparing the ESD procedure time by lesion location, the median ESD procedure time of S–O clip-assisted ESD was significantly shorter than that of conventional ESD in the upper and middle third of the stomach (39.4 min *vs* 58.3 min; P = 0.005). In the lower third of the stomach, the two methods were not significantly different (22.8 min *vs* 36.2 min; P = 0.146). Essentially, the ESD procedure performed in the lower third of the stomach is easier than that performed in the upper and middle third of the stomach[60,61]. The difference in the difficulty of the ESD procedure may explain the difference in the subgroup analysis outcomes. Therefore, the S–O clip-assisted ESD is especially recommended for lesions in the upper and middle third of the stomach. Meanwhile, en bloc resection, R0 resection, perforation, and post-ESD bleeding showed no significant difference between the two groups. The S–O clip slip-off rate was only 2.5%, probably because the modified attachment method prevented interference between the endoscope and spring part of the clip. In this trial, vertical traction was selected for the S–O clip-assisted ESD using its multidirectional traction function. Considering this result and the result of subgroup analysis of the above-mentioned multicenter randomized controlled trial of the clip-with-line method, vertical traction may be the optimal traction direction for gastric ESD. Although other internal traction methods, including the pulley, double scope, and magnetic anchor methods, may be able to provide vertical traction, their feasibility in gastric ESD is unclear and needed to be assessed.

The usefulness of the sheath traction method in gastric ESD has been reported. Unlike the clip-with-line method, the sheath traction method allows traction not only in the pulling direction but also in the pushing direction, so distal traction can be avoided in both forward and retroflexed endoscopic positions. A retrospective study comparing conventional ESD (n = 20) and the clip-and-snare method (n = 20) demonstrated that the clip-and-snare method significantly reduced the median ESD procedure time (38.5 min *vs* 59.5 min; P = 0.023)[29]. En bloc resection was achieved without perforation in all the patients in both groups. A case series of 21 challenging gastric ESD cases treated using the Endo Trac reported that the ability to change the traction direction in both proximal and distal sides was 100%[31]. Although these results are promising, the stress on the operator due to possible interference between the sheath and the endoscope is concerning. Moreover, these studies have the limitations of being retrospective studies with small numbers of cases. Therefore, evaluation in large-scale studies is warranted.

ESTD was reported to be useful not only for esophageal ESD but also for gastric ESD. A retrospective study evaluating 799 consecutive cases of gastric ESD in single institution showed that resection speed using ESTD was greater than with conventional ESD (19.3 mm^2/min *vs* 17.7 mm^2/min; P = 0.009)[62]. Perforation was significantly less frequent in ESTD (0.9% *vs* 6.0%; P = 0.035). However, the creation of a submucosal tunnel in the stomach may be more difficult compared to the esophagus because the stomach lumen is not straight. It has been reported difficult to form a submucosal tunnel for a lesion located at the pylorus ring or the greater curvature side of the fornix[62]. On the other hand, lesions located at the cardia, the lesser curvature of the gastric corpus, and the greater curvature of the antrum are reported to be suitable for ESTD[63].

In summary, the internal traction method using the S–O clip with modified attachment method has the potential to be the most appropriate traction method for

gastric ESD. The clip-with-line method and ESTD may be effective methods in gastric ESD if the lesion location is appropriate for these methods.

Colon and rectal ESD

Colonic ESD is more challenging than esophageal and gastric ESD because the maneuverability of the endoscope is limited, the colorectal lumen is angulated, and the muscle layer is thin and easy to perforate. Withdrawal and reinsertion of the endoscope is time-consuming in colonic ESD, unlike ESD in the upper gastrointestinal tract and rectum. Therefore, for colonic ESD, a traction method that does not require withdrawal and reinsertion of the endoscope is suitable. By contrast, it is easy to utilize most traction methods in rectal ESD.

Internal traction methods are suitable not only for rectal ESD but also colonic ESD because they do not require withdrawal and reinsertion of the endoscope. Recently, several novel devices for internal traction in colorectal ESD have been reported, such as S–O clip[7,8], ring thread[47], multiloop[48], double clip and rubber band[49], and clip band[50]. These devices have the common advantage of controlling traction direction at anchor site. Among them, the S–O clip is made of a highly elastic spring that can be used flexibly, regardless of the lesion location. A prospective randomized controlled trial comparing conventional (n = 27) and S–O clip-assisted ESD (n = 23) demonstrated that the mean ESD procedure time for S–O clip-assisted ESD was significantly shorter than that for conventional ESD (37.4 min *vs* 67.1 min; P = 0.03)[64]. No significant differences were observed in en bloc resection, perforation, and post-ESD bleeding. Although the conventional ESD was converted into the S–O clip-assisted ESD in eight cases, these cases remained in the conventional ESD group. In most of these conversion cases, the lesions were located in flexural areas where endoscope maneuverability is poor; these areas were the sigmoid colon, hepatic flexure, and splenic flexure. In these areas, reaching under the mucosal flap by the endoscope tip is difficult when only used with hood traction. The S–O clip helps the endoscope tip reach under the mucosal flap, providing proper visualization of the submucosa, despite poor endoscope maneuverability. Traction-assisted ESD using ring thread[47], multiloop[65], or double clip and rubber band[49] also showed promising treatment results in clinical trial, compared with conventional ESD. Further studies should focus on which traction direction is appropriate for colorectal ESD by using multidirectional traction function of internal traction methods.

PCM is another traction method that does not require withdrawal and reinsertion of the endoscope. In this method, the submucosal pocket holds the endoscope, allowing stable endoscope maneuverability. Moreover, the endoscope inside the submucosal pocket pushes up the lesion and provides sufficient tension at the dissection plane. A prospective randomized controlled trial comparing PCM (n = 59) and the conventional method (n = 55), conducted at three Japanese institutions, reported that the rate of ESD completion (defined as completion of colorectal ESD in three hours with en bloc resection using the assigned ESD method without changing to other methods or other devices and without perforation during the procedure) was significantly higher in PCM compared with conventional ESD (93% *vs* 73%; P = 0.01)[66]. By contrast, the median dissection speed was not significantly different between the two methods (15.9 mm^2/min *vs* 17.4 mm^2/min; P = 0.81). This was unforeseen, as several retrospective studies had reported dissection speed significantly greater in PCM than in the conventional method[67,68]. A novel method that combines the PCM and internal traction has been developed[52,53], and it can possibly accelerate the dissection speed. A meta-analysis including five studies (two randomized controlled trials and three retrospective studies) evaluated the efficacy and safety of PCM in comparison with the conventional method for superficial colorectal neoplasms[69]. PCM achieved a higher R0 resection rate (93.5% *vs* 78.1%; OR, 3.4; 95%: 1.3–8.9; I^2 = 58%), a higher en bloc resection rate (99.8% *vs* 92.8%; OR, 9.9; 95%CI: 2.7–36.2; I^2 = 0), a shorter procedure time (min) [mean difference (MD), -11.5; 95%CI: -19.9 to -3.1; I^2 = 72%], a faster dissection speed (mm^2/min) (MD, 3.6; 95%CI: 2.8–4.5; I^2 = 0), and a lower overall adverse event rate (4.4% *vs* 6.6%; OR, 0.6; 95%CI: 0.3–1.0; I^2 = 0) than the conventional method. However, all the included studies were conducted in Japan, with only two randomized controlled trials. Hence, further study is needed, especially regarding dissection speed.

The conventional clip-with-line method requires withdrawal and reinsertion of the endoscope, which may be troublesome during colonic ESD for lesions located where it is difficult to insert the endoscope. Modified preparation techniques for the clip-with-line method have been developed that eliminate withdrawal and reinsertion of the endoscope[70]. A single-center prospective randomized controlled trial comparing the clip-with-line method with modified preparation technique (n = 42) against the

conventional method (n = 42) demonstrated that the median colorectal ESD procedure time was significantly shorter in the modified clip-with-line method than in the conventional method (40 min *vs* 70 min; P < 0.0001)[71]. No significant differences were noted in en bloc resection, R0 resection, perforation, and post-ESD bleeding. In this study, two experts and two intermediates performed the colorectal ESD procedures. When the intermediates encountered difficult situations, the experts took over the procedure. The intermediates' self-completion rate was significantly higher in the modified clip-with-line method than in the conventional method (100% *vs* 90%; P = 0.04). Although this modified preparation technique is a little tricky, and the clip-with-line method is not able to control the traction direction, it has the advantage that does not require any special device. Since colorectal ESD is generally performed in the forward endoscopic position, the clip-with-line method may provide diagonally proximal or proximal traction for colorectal ESD. The clip-with-line method may be effective, as long as the endoscope has a parallel approach to colorectal wall.

The sheath traction, clip-and-snare, and Endo Trac methods also requires withdrawal and reinsertion of the endoscope. However, the sheath traction method was reported to be utilized even for colonic lesions[27,31]. A retrospective study reported that the clip-and-snare method (n = 17) significantly reduced mean colorectal ESD procedure time compared with conventional method (n = 123) (45.6 min *vs* 70.1 min; P = 0.047)[28]. There were no significant differences in en bloc resection, curative resection and adverse events (perforation and post-ESD bleeding). The sheath traction method has the great advantage that it can control traction direction to some extent by pushing or pulling the sheath, which may facilitate the ESD procedure. Moreover, the clip-and-snare method may be useful in any country, because it does not require any special device. Although reinsertion of the endoscope during the sheath traction method is occasionally troublesome, a balloon overtube may help address the issue, simplifying insertion of the endoscope.

The usefulness of underwater techniques during colorectal ESD has been reported [20,72-75]. Underwater conditions provide buoyancy (classified as natural traction) which can help turn over the mucosal flap of a lesion located lower gravitationally (Figure 7C and D). Although colorectal ESD is generally performed with the patient's posture such that the target lesion is on the upper side of gravity, to open the mucosal flap by gravity, it is difficult to select this posture in some cases due to poor endoscope maneuverability, a vertical approach to the lesion, and difficulty opening the lumen. Water pressure from the endoscope using its water supply function can be used as a traction method at any time. Water pressure can be used even in the conventional method. However, splashing can sometimes obstruct the visual field. In underwater conditions, splashing can be avoidable, which makes it easier to get under the mucosal flap. The underwater condition provides a good field of vision through a zoom effect and the disappearance of halation; this facilitates colorectal ESD in a poor field of vision due to severe submucosal fibrosis or fat tissue (Figure 16). We reported a case series study that demonstrated the feasibility and safety of underwater techniques for colorectal ESD[20]. However, additional studies are needed to evaluate whether underwater techniques improve colorectal ESD procedures compared with conventional methods.

In conclusion, PCM and internal traction methods (*e.g.*, S–O clip, ring thread, multiloop, double clip and rubber band, clip band) are especially recommended for colorectal ESD, based on the results of recent studies.

Duodenal ESD

Duodenal ESD is extremely challenging due to the fragile muscle layer, thin submucosal layer, and poor maneuverability of the endoscope, resulting in a high perforation rate which has been reported as 8.8% to 27.0%[76-78]. However, there are few reports of effective methods for reducing the procedural difficulty of duodenal ESD, because superficial duodenal epithelial tumors indicated for ESD are rare.

PCM has been reported to be useful for the treatment of superficial duodenal epithelial tumors. A retrospective study showed that perforation was significantly less frequent in PCM [7% (2/28)] than in the conventional method [29% (5/17; P = 0.046)] [79]. PCM demonstrated a faster dissection speed (9.4 mm^2/min *vs* 6.5 mm^2/min; P = 0.09) and a higher en bloc resection rate (100% *vs* 88%; P = 0.07) than the conventional method, although the differences were not statistically significant. PCM may prevent leakage of the injected solution from the submucosa due to minimal mucosal incision until completion of submucosal dissection. Moreover, the submucosal pocket holds the endoscope, providing stable endoscope maneuverability. In the conventional method for duodenal ESD, the mucosa around the lesion is incised widely before the completion of submucosal dissection, while the injected solution in the submucosa may

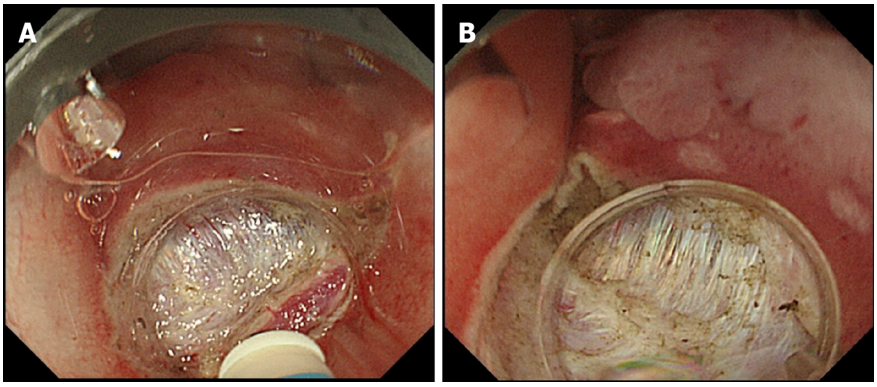


Figure 16 Comparison of aerial and underwater endoscopic view. A: Severe fibrosis and halation makes the boundary between the submucosal layer and muscle layer unclear; B: Submergence enables a detailed observation through a natural zoom effect and causes halation disappearance, thereby clarifying the boundary between the submucosal layer and muscle layer. Citation: Nagata M. Usefulness of underwater endoscopic submucosal dissection in saline solution with a monopolar knife for colorectal tumors (with videos). *Gastrointest Endosc* 2018; 87(5): 1345-1353. Copyright © 2018 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[20].

easily flow out and the endoscope operability deteriorates.

Underwater techniques are another option for the treatment of superficial duodenal epithelial tumors[21,80]. Severe submucosal fibrosis in duodenal submucosa occasionally exists and causes insufficient submucosal elevation, even with a large quantity of injection. Although submucosal fibrosis generally makes it difficult to get under the mucosal flap during the first half of duodenal ESD, water pressure from the endoscope using its water supply function helps turn over the mucosal flap and enable the endoscope to get under the mucosal flap. Since the underwater condition eliminates splashing, unlike under gas supply conditions, the endoscope can get under the mucosal flap seamlessly after generating water pressure. This technique is classified as natural traction, and can be used repeatedly at any time.

The underwater condition has several useful effects based on nature, such as a zoom effect, the disappearance of halation, buoyancy, and a heat sink effect. The zoom effect and the disappearance of halation allow recognition of the proper dissection plane despite severe submucosal fibrosis. Buoyancy can be classified as natural traction and aids the opening of the mucosal flap when the lesion is gravitationally lower (Figure 7C and D). The heat sink effect minimizes thermal damage to the muscle layer from the ESD procedure. Thermal damage may increase the risk of delayed perforation after duodenal ESD, which leads to serious complications[81]; therefore, the underwater techniques may be suitable for duodenal ESD.

The underwater techniques may be able to be combined with other traction methods. In fact, a case report showed the usefulness of the underwater techniques with PCM and internal traction during duodenal ESD[82]. Although underwater techniques have major disadvantages, such as visual field loss due to active bleeding, gel immersion endoscopy, which secures the visual field during bleeding, may help address this issue[83]. Underwater techniques have the potential to reduce difficulty in ESD procedures through unique effects not found in conventional methods under gas supply condition. Further study should focus on the efficacy of ESD with underwater techniques, especially in the duodenum.

Several traction methods other than PCM and underwater techniques for duodenal ESD have been reported, such as internal traction using the S–O clip[84] and the sheath traction method[31]. However, there are few reports about these methods, and the efficacy of these methods is still unclear.

In summary, PCM and underwater techniques have the potential to facilitate the duodenal ESD procedure, decreasing the risk of perforation.

CONCLUSION

The purpose of traction during ESD is to create a visual field by turning over the mucosal flap and facilitate dissection by providing tension for the dissection plane. In order to achieve these effects, it is important to understand the advantages and disadvantages of each traction method and to use a traction method that is most appropriate as per the situation. The results of previous studies suggest that traction

direction affects the effectiveness of the traction method. Therefore, traction direction should be considered when choosing a traction method. Although there are increasing reports of methods that can control the traction direction, further studies should focus on investigating the optimal traction direction and its influence on the effectiveness of the traction method.

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FRONTIER

Cytoprotective gastric pentadecapeptide BPC 157 resolves major vessel occlusion disturbances, ischemia-reperfusion injury following Pringle maneuver, and Budd-Chiari syndrome

Predrag Sikiric, Anita Skrtic, Slaven Gojkovic, Ivan Krezic, Helena Zizek, Eva Lovric, Suncana Sikiric, Mario Knezevic, Sanja Strbe, Marija Milavic, Antonio Kokot, Alenka Boban Blagaic, Sven Seiwerth

ORCID number: Predrag Sikiric 0000-0002-7952-2252; Anita Skrtic 0000-0002-9275-7283; Slaven Gojkovic 0000-0003-4020-326X; Ivan Krezic 0000-0001-7994-5645; Helena Zizek 0000-0001-9863-4164; Eva Lovric 0000-0003-3346-7005; Suncana Sikiric 0000-0001-9337-9248; Mario Knezevic 0000-0001-6448-7274; Sanja Strbe 0000-0001-7199-4530; Marija Milavic 0000-0003-3404-0644; Antonio Kokot 0000-0002-4836-7536; Alenka Boban Blagaic 0000-0003-1266-3402; Sven Seiwerth 0000-0002-5894-419X.

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Predrag Sikiric, Slaven Gojkovic, Ivan Krezic, Helena Zizek, Mario Knezevic, Sanja Strbe, Alenka Boban Blagaic, Department of Pharmacology, School of Medicine, University of Zagreb, Zagreb 10000, Croatia

Anita Skrtic, Eva Lovric, Suncana Sikiric, Marija Milavic, Sven Seiwerth, Department of Pathology, School of Medicine, University of Zagreb, Zagreb 10000, Croatia

Antonio Kokot, Department of Anatomy and Neuroscience, Faculty of Medicine Osijek, J.J.Strossmayer University of Osijek, Osijek 31000, Croatia

Corresponding author: Predrag Sikiric, MD, PhD, Full Professor, Department of Pharmacology, School of Medicine, University of Zagreb, Salata 11, Zagreb 10000, Croatia. sikiric@mef.hr

Abstract

The stable gastric pentadecapeptide BPC 157 counteracts various venous occlusion-induced syndromes. Summarized are all these arguments, in the Robert's cytoprotection concept, to substantiate the resolution of different major vessel occlusion disturbances, in particular ischemia-reperfusion injury following the Pringle maneuver and Budd-Chiari syndrome, which was obtained by BPC 157 therapy. Conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance (the recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). In this paper, we summarize the evidence of the native cytoprotective gastric pentadecapeptide BPC 157, which is stable in the human gastric juice, is a membrane stabilizer and counteracts gut-leaky syndrome. As a particular target, it is distinctive from the standard peptide growth factors, involving particular molecular pathways and controlling VEGF and NO pathways. In the early 1990s, BPC 157 appeared as a late outbreak of the Robert's and Szabo's cytoprotection-organoprotection concept, like the previous theoretical/practical breakthrough in the 1980s and the brain-gut axis and gut-brain axis. As the time went on, with its reported effects, it is likely most useful theory practical implementation and justification. Meantime, several reviews suggest that BPC 157, which does not have a lethal dose, has profound cytoprotective activity, used to be demonstrated in ulcerative colitis and multiple sclerosis trials. Likely, it may bring the theory to practical application, starting with the

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initial argument, no degradation in human gastric juice for more than 24 h, and thereby, the therapeutic effectiveness (including *via* a therapeutic per-oral regimen) and pleiotropic beneficial effects.

Key Words: Gastric pentadecapeptide BPC 157; Cytoprotection; Major vessel occlusion disturbances; Pringle maneuver; Budd-Chiari syndrome; Therapy

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Core Tip: Summarizing the essential epithelium and endothelium protection interplay described in Robert's and Szabo's cytoprotection concept, and the role of the stable pentadecapeptide BPC 157 as a likely mediator, we suggest that BPC 157 may be a useful cytoprotective therapy. The hope is that it could finally bring into practice the huge theoretical importance of all aspects of the cytoprotection concept. Conceptually, there is a new point to discuss, namely, endothelium maintenance to epithelium maintenance (recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). BPC 157 counteracts various venous occlusion-induced syndromes, as well as inferior caval vein syndrome, ischemia-reperfusion injury following Pringle maneuver, and Budd-Chiari syndrome in rats. Activation of the alternative collateral pathways to bypass occlusion and reestablish alternative blood flow, results in the counteraction of the consequent syndromes. The severe venous occlusion-induced disturbances, the high portal and caval hypertension, aortal hypotension, arterial and venous thrombosis, both peripherally and centrally, and various organ lesions (*i.e.*, gastrointestinal, liver, kidney, heart, and brain) were all attenuated and/or eliminated. Furthermore, this particular beneficial effect may be competing with the Virchow's triad that can be a common presentation [*i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, bile duct ligation-induced liver cirrhosis and portal hypertension, temporary portal triad occlusion (ischemia-reperfusion injury following the Pringle maneuver), and suprahepatic occlusion of the inferior caval vein (Budd-Chiari syndrome)]. The resolution of these various venous occlusion-induced syndromes emphasizes the evidence that as the native cytoprotective gastric peptide and a stable gastric pentadecapeptide membrane stabilizer, BPC 157, which is stable in the human gastric juice and counteracts gut-leaky syndrome, is a particular target and easily distinguished from standard peptide growth factors, involving particular molecular pathways, and specifically controlling the VEGF and NO pathways, in particular the prostaglandin pathway.

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INTRODUCTION

The current review aims to evaluate whether the stable gastric pentadecapeptide BPC 157, which has consistent efficacy in the co-, pre-, and post-treatment regimens, with a rapid onset of the therapeutic effect, as well as the parenteral and per-oral effectiveness, may bring the Robert's cytoprotection theory into practical application[1].

As previously stated[1], all of the studies to date that have tested the stable gastric pentadecapeptide BPC 157 as a treatment have demonstrated extremely positive healing effects for various injury types in numerous organ systems. Its practical significance as a prototypic cytoprotective agent and an important mediator of Robert's cytoprotection[1], and its contribution to resolving Selye's stress response[1], and brain-gut and gut-brain axis activity have been reported[2]. Additional particular points are its wide interactions with the nitric oxide (NO) system[1] and prostaglandin

system and counteraction of the toxicity of non-steroidal-anti-inflammatory drugs[1]. Its therapeutic effects on fistula healing[1] and damaged skin, muscles, tendons, ligaments, and bone comparable to those in the gastrointestinal tract[3,4] and wound healing, in particular[3], are also reviewed. The counteraction of tumor-induced muscle cachexia and the signaling process implicated in cancer cachexia[5] and leaky gut, and its membrane stabilizer and free radical scavenger activity[6] are highlighted. The final focus is on the particular effect of BPC 157 on blood vessels and vessel recruitment[1,7]. In addition, BPC 157, due to its profound cytoprotective activity, which has been demonstrated in ulcerative colitis and applied to multiple sclerosis trials, may be used, since it does not have a lethal dose (LD1)[1,6]. In one of the most recent studies[3], BPC 157 was found to be distributed in the gastrointestinal mucosa, lung, bronchial epithelium, epidermal layer of the skin, and kidney glomeruli by *in situ* hybridization and immunostaining. These data suggest that BPC 157 may have additional regulatory roles in the function of the lungs, kidneys, and skin in humans, in addition to being isolated from gastric juice and primarily acting in the gastrointestinal system[3]. BPC 157 has also been reviewed in several other articles[8].

In the present review, we discuss the cytoprotective activity of the gastric pentadecapeptide BPC 157[1] to resolve major vessel occlusion disturbances, ischemia-reperfusion injury following the Pringle maneuver, and Budd-Chiari syndrome[9-11], providing evidence that it may bring the cytoprotection theory to practical application. On the other hand, as mentioned above, the stable gastric pentadecapeptide BPC 157 perfectly matched with the original Robert's cytoprotective requirements for the stomach, or even extended it[1]. These requirements are the protection of the epithelium ("epithelial pathway") and endothelium ("endothelial pathway"), and the maintenance of gastrointestinal mucosal integrity to obtain a large beneficial effect inside and outside the gastrointestinal tract. Typically, human gastric juice rapidly destroyed standard growth factors within 15 min[12,13]. In contrast, BPC 157, with its essential gastric juice origin and stability in human gastric juice for more than 24 h [12], was matched in the local level (stomach, the permanent maintenance of the mucosal integrity, and thereby the entire gastrointestinal tract)[1]. Therefore, BPC 157 has particular therapeutic effectiveness, including *via* a therapeutic per-oral regimen, and pleiotropic beneficial effects. This local stomach and gastrointestinal tract protection was further extended to the general level (protection of other organs) (cytoprotection to organoprotection)[1]. As previously mentioned, BPC 157 could follow both the "epithelial" and "endothelial" pathways in Robert's cytoprotection[1,7, 12,14].

According to Andre Robert[14] in 1975, the first indication for cytoprotection was the evidence that certain prostaglandins (PGF2 and PGFB, which could not affect gastric acid secretion) protected the gastric mucosa against indomethacin, in a gastric acid-independent manner, *via* a mechanism other than the inhibition of gastric acid secretion[14-16]. Therefore, the term *cytoprotection* pioneered by Robert[14-16] was introduced in 1979 against the noxious effect of both intragastric alcohol application and the use of nonsteroidal anti-inflammatory drugs (NSAIDs). According to Robert's concept, the rapid onset of gastric cytoprotection would be the most remarkable aspect [14-16]. Prostaglandins reduce the development of gastric necrotic lesions when given orally at an appropriate dose as late as 1 min before the administration of absolute ethanol. Knowing this "before but not after effectiveness" as a limitation, Robert appreciated the curing of the already existing lesions as the further possibility[14-16]. The full explanation was provided in a few subsequent reviews[14-16] and the full argument was later substantiated[14-16]. In our view of the new cytoprotection principle, the essential evidence of Robert (epithelial protection) is the remarkable ability of endogenous and exogenous cytoprotective agents (*i.e.*, prostaglandins) to prevent rapidly acute gastric hemorrhagic lesions induced by diverse noxious stimuli such as ethanol, bile acids, hyperosmolar solutions, and NSAIDs such as aspirin or indomethacin. According to the claims of Robert [*i.e.*, cytoprotection preventing mucosal necrosis caused by noxious agents due to the direct damage of cells or a local deficiency of cytoprotective mediators (*i.e.*, prostaglandins)], the cytoprotection concept also goes beyond peptic ulcer therapy[14-16]. Moreover, in Robert's view[14], the demonstration of adaptive cytoprotection suggests that cytoprotection by prostaglandins may be a physiological phenomenon[14]. One milliliter of 20% ethanol (as small irritant) given orally (note, in Robert's publication, "orally" implies administration *via* a tube into the stomach, or rather intragastric application, see Chapter *2.1.3. Epithelial pathway to adaptive cytoprotection*) to fasted rats 15 min prior to giving absolute ethanol (regarded as a strong irritant) prevented the gastric mucosal necrosis caused by the latter[17]. For the effectiveness of cytoprotective agents, the concept holds the protection of stomach cells (cytoprotection) and other epithelia (organoprotection)

against direct injury to cells induced by various noxious agents[14-16]. In addition, see the notation for the cytoprotection-organoprotection for other agents[18,19].

In the early 1980s, the concept obtained an additional key, the concomitant protection of the stomach endothelium[20,21] or Szabo's vascular injury, as an early pathogenic factor in the development of ethanol-induced gastric hemorrhagic erosions. Demonstration of the vascular injury was seen within 1 min, as was the estimated effect of the agents[20,21]. This vascular point in ethanol-induced gastric lesions was fully elaborated in a series of subsequent reports[22]. Since then, the rapid recovery of damaged endothelium may be considered a shared effect of the cytoprotective agents [23].

Thus, the cytoprotection theory holds that cytoprotective agents should exert direct epithelial and endothelial cell protection inside and outside of the gastrointestinal tract [14-16], *via* the "epithelial pathway" and "endothelial pathway". There is an essential evolvement in the stomach protection: Endothelium protection to epithelium protection[14-16]. Each of these pathways originates as a result of the increase in cytoprotective activity, together manifested as an increased therapeutic effect in both the prophylactic process (important for maintaining undisturbed organ function) and the therapeutic process (important for the possible reversal of the damaged tissue to a normal structure, and the interruption of damaging events). Unfortunately, such practical realization of the highly conceptualized theory is lacking. The anticipated huge range of organ lesions that should be counteracted and protection against non-specific lesions[1], as well as the rapid onset of action implemented in the agents' efficacy, as a resolving outcome, remains obscure.

On the other hand, in addition to the proposed role of BPC 157[1,7], within Robert's concept of cytoprotection, different points of view and different highlights can be clearly seen. Certainly, such a multitude illustrates the essential value of the potential application of the concept. The emphasis was on NO, carbon monoxide and hydrogen sulfide[24-26], sulfhydryls (SH)[20,27,28] in parallel with prostaglandins, as well as histamine[23], prostaglandins[17], EP1 and IP receptors[29,30], the healing action of antacid[31], sucralfate[31,32], heat shock protein 70 (HSP70)[33], and the renin-angiotensin system and active angiotensin metabolites[34]. Further illustrative emphases include opioids[35], alpha-2 adrenoreceptors[36], glucocorticoids[20,37-39], thyrotropin-releasing hormone (TRH)[40-43], capsaicin[44-46], dopamine[19,47-51], somatostatin[18,52,53], epidermal growth factor (EGF)[53-55], bombesin[56], ghrelin [17,34,57], cholecystokinin (CCK) and leptin[58,59], melatonin[60], neurotensin[61], fibroblast growth factor (FGF)[62,63], agmatine[64], amino acids[65], second-generation histamine H(2)-receptor antagonists[66], hemeoxygenase-1[67,68], and the molecular basis of alcohol-related gastric and colon cancer (acetaldehyde)[69].

Finally, a historical cytoprotection review, along with many original details, is given by Mozsik[70].

The term cytoprotection was commonly coined in other organ studies, *i.e.*, the heart and brain[71,72], kidney[73], liver[74], eye[75], skin and wounds[76], bone[77], and skeletal muscle[78].

Unfortunately, the multitude of agents supposed to be involved did not resolve the conceptual problems that were initially shown with the prime agents, providing the limited therapeutic potential of prostaglandins in stomach lesions (*i.e.*, prostaglandins might only prevent rather than cure any already established stomach lesions)[14-16]. Likewise, there was an even more limited therapeutic potential in the healing of other organs (prostaglandins were only effective in a few organ lesions)[79-81]. The switching to other cytoprotective agents (*i.e.*, sulfhydryls[19,21], somatostatin[18], EGF [53], TRH[41,67,82], opioids[83], dopamine[50,51,61], and CCK) led to similar incomplete results in both stomach and other organ lesions (for review see[1,7]). Consequently, considering the application and efficacy of standard agents, a considerable gap remains between the theoretical potential and practical realization[1,7]. Considering the supplemental endothelium protection, after initial demonstration in the stomach, no endothelial protection outside the stomach was investigated at the time[22]. Of note, BPC 157 appears to resolve both of these issues, *i.e.*, the "epithelium pathway" and "endothelium pathway" in cytoprotection[1,7], and may both prevent lesion development and cure any established lesions.

Likewise, to illustrate the failed realization of the concept with standard antiulcer agents, in addition to only prophylactic effectiveness in stomach lesions and a few other organs in which effectiveness was shown, the theoretical/practical problem is that standard cytoprotective agents also demonstrated the opposite outcome[42,84]. The intriguing point is the sulfhydryl prototype, cysteamine, and sulfhydryl conceptual involvement[19,21]. Cysteamine is highly protective in alcohol-induced stomach lesions[19,21], but, in contrast, cysteamine application provided the most

valuable standard model for the induction of duodenal[85,86] and colon[87] lesions. Also, we emphasized[1] that Robert's concept[14-17] largely applied the antecedent Selye's stress concept[88,89] which essentially contributed[90] to the introduction of corticosteroid therapy[91]. Of note, both concepts act against various noxious non-specific agents that would induce non-specific lesions[1]. Both concepts also hold organoprotection (Selye's concept of homeostasis that should be reestablished by the stress response[88] *vs* Robert's direct stomach cell protection that should be generalized by the application of cytoprotective agents[14-16]), and adaptation [Selye's small stress that protects against severe stress[89] *vs* Robert's small irritants that protect against strong irritants (adaptive cytoprotection[17])]. However, the essential first mediator of Selye's stress concept[88,89], which would integrate the adaptive bodily stress response and reestablish organoprotective bodily homeostasis, remained undiscovered, and appeared to be a major weakness of the concept that would preclude its practical realization[92,93].

For the classic concepts of Robert and Selye[14-16,88], the adverse effects of the prototype agents (*i.e.*, mediators) appeared to be an additional pitfall. Obviously, protection against direct injury to the cell in Robert's cytoprotection concept[14-16] certainly precludes any adverse effects, which are quite common with the application of prostaglandin analogues[65]. Likewise, the reestablishing of homeostasis (Selye's stress response defined "as such")[88] does not include the adverse effects that have been commonly known for the application of corticosteroids since early times[94]. As BPC 157 appears to be very safe and LD1 was not achieved, with no side effects reported in clinical trials, the possible switching of beneficial effects to negative ones (over-shutting phenomenon) appears to be highly unlikely[1].

However, whatever the pitfalls may be, these two concepts[14-16,88] provided a firm theoretical frame for the development of novel agents and therapies. It should be practically realized and demonstrated, in addition to the local (stomach) beneficial effect, by the agents' pleiotropic beneficial effects[1,7]. If properly followed, it may fulfill the first conceptual beneficial point (starting with Robert's cytoprotection[14-16], local protection and therapy of the stomach and gastrointestinal tract achieved) by the next extended beneficial point (the protection of other organs [epithelia] and the achievement of therapy), and bring them together to a reality that can no longer be disputed.

Thus, in the early 1990s, pentadecapeptide BPC 157[1,7] appeared as a late outbreak of the cytoprotection-organoprotection concept of Robert and Szabo[14-16,18,19], for epithelial and endothelial protection, like the previous theoretical/practical breakthrough in the 1980s[14-16,18,19] and the brain-gut axis and gut-brain axis[3]. As time went on, with its reported effects, BPC 157 could be most useful in the practical implementation and justification of the theory[1]. All arguments were given to bring the long-standing theory into practice, starting with the initial argument of the lack of degradation in human gastric juice for more than 24 h[12], and thereby the therapeutic effectiveness (including *via* a therapeutic per-oral regimen) and pleiotropic beneficial effect[1,7].

BPC 157 IN CYTOPROTECTION

Overall, and in particular for the role and cytoprotective effectiveness of BPC 157, it is safe to speculate that the efficacy and activity limitation of this agent, and thereby its practical application, would be determined by the foundation of the standing concepts, and *vice versa*. Briefly, the agent "runs" within the concept frame, and *vice versa*. Ideally, agent and concept can match completely (as may be seen with the achieved extent of the obtained beneficial effects; cytoprotection can be manifested as a huge range of beneficial effects, both inside and outside the gastrointestinal tract). General pitfalls may be the number of mentioned cytoprotective agents that have previously failed to match the required cytoprotection concept. In general, this means more problems with the use of new agents, and more problems for the concept to maintain its validity and less possibility (enthusiasm and belief) to be once applied. Alternatively, if there were no known agents which fulfilled the requirements of the standing concepts, the agent's efficacy and activity would determine the opposition to the "law" of the standing concepts and form a new relevant concept.

Illustratively, regarding the sympathetic system function, Alhquist's receptor concept[95] (*i.e.*, six catecholamines, and their different order of potency depending on the tissue involved, to anticipate the presentation of the particular alpha and beta receptors) discharged the long-standing "law" of physiology, Cannon's concept of two

mediator substances (sympathin E and sympathin I)[96]. Although there was an overlap of several years, Alhquist's receptor concept accuracy[95] envisaged the development of specific blocking agents in the subsequent years[97], and the consequent regular use of beta blockers in a large range of indications[98]. However, similar general acceptance and applicability did not arrive for the cytoprotection concept, nor was there any proof[99,100], when years later, as alternative gastric acid-nondependent, Robert's cytoprotection theory challenged the peptic ulcer therapy[14-16]. The lack of a practical solution and the absence of any commonly applicable cytoprotective therapy[99,100] mean that the "law" "no acid-no ulcer" and the superiority of H2-blockers were not discharged until the present time[101].

In the early 1990s, BPC 157 was introduced as a pentadecapeptide with cytoprotective effects[1,18,19], many years after the breakthrough of the original concepts of Robert[14-16] and Selye[88]. The surveillance of these two major concepts[14-16,88] and their development and achievements lacking full realization and adequate practical application[92,93,99,100] considered the introduction of BPC 157 to be too late a challenge. Seeing from the achieved perspective of all agents tested as standard cytoprotective agents, it was safe to speculate that a novel agent would hardly achieve a wider range of pleiotropic beneficial effects and drug characteristics that remained elusive for years.

However, conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance is upgraded to endothelium maintenance to epithelium maintenance (collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel)[1,7]. The recruitment of collateral blood vessels would compensate for vessel occlusion and reestablish blood flow[1,7,9-11]. BPC 157 counteracted various venous occlusion-induced syndromes[9-11], inferior caval vein syndrome[9], ischemia-reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in rats. This beneficial effect was also shown for other syndromes, *i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, and bile duct ligation induced liver cirrhosis and portal hypertension[1,7]. The resolution of these various venous occlusion-induced syndromes[1,7,9-11] emphasized the practical evidence. The stable gastric pentadecapeptide BPC 157, as a membrane stabilizer[5], likely acts as the native cytoprotective gastric peptide[1,3,7], which is resistant and stable in human gastric juice[12], and counteracts gut-leaky syndrome[6]. As a particular target, it is distinct from the standard peptide growth factors[3], involving particular molecular pathways [102-105], particularly controlling VEGF and NO pathways[1,106,107], and the prostaglandin pathway[1].

Epithelial pathway in stomach and gastrointestinal tract healing for cytoprotection against direct cell injury produced by direct contact with noxious agents

BPC 157 consistently counteracted the gastric lesions induced by 96% alcohol[1]. Of note, epithelial protection, as direct cytoprotection against direct cell injury produced by direct contact with noxious agents (*i.e.*, alcohol)[14-16], appears to be essential to resolve the follow-up of Robert's stomach cytoprotection ("epithelial pathway")[14-16]. As with Robert's alcohol intragastric application, this was a more advantageous therapeutic effect, overriding previous common limitations shared by standard cytoprotective agents (*i.e.*, prophylactic effect that may only counteract lesion development, but is unable to cure already existing lesions upgraded to the equal therapeutic ability[1]). BPC 157 demonstrated very consistent efficacy in alcohol-induced gastric lesions for co-, pre-, and post-treatment regimens, with a rapid onset of therapeutic effect, thereby providing consistent evidence for undistributed pertinent and specific effects, such as protection and healing, and the likely positive effects of an unusually high range[1]. This essential stomach point is confirmed and appreciated by others[108]. The BPC 157 equipotent (co-, pre-, and post-treatment regimens, per-oral and parenteral) beneficial effect is particular. There are constant interactions with the NO system and capsaicin-sensitive somatosensory neurons, since it consistently appears in naive rats as well as in those challenged with NOS blockade (NOS blocker L-NAME), NOS substrate L-arginine (NOS over-activity), NO system immobilization (concomitant application of L-NAME and L-arginine), capsaicin (as newborn or adult), or small exogenous or endogenous irritants[1,7]. A comparable beneficial effect was also achieved *in vitro* (denervated (isolated) gastric mucosal cells)[109,110].

Further supporting evidence included a strong reduction of the Monastral blue staining in ethanol-treated rats and, thereby, endothelium maintenance[1,7] and comparable beneficial effect in the stress gastric ulcer model[1] and cysteamine-duodenal ulcer model[1]. The same high efficacy was observed for both intragastric

and intraperitoneal regimens[1,7]. The evidence that BPC 157 fully counteracted NSAID-induced gastric and intestinal lesions is consistent with the prostaglandin requirement of Robert's model, and the beneficial effect of BPC 157 in the entire gastrointestinal tract[1]. Also, in addition to cysteamine- or ischemia/reperfusion-induced colitis[1,7], BPC 157 counteracted trinitrobenzene sulfonic acid (TNBS)[111] or iodoacetamide[112,113]-induced ulcerative colitis. Of note, the beneficial effect of BPC 157 is long-lasting, and may also counteract ulcer recidivation (*i.e.*, cysteamine ulcerative colitis)[1,7]. Also important for the issue of cytoprotection is the evidence that BPC 157 may counteract stomach ulcer and induce ulcer regression (*i.e.*, clopidogrel-induced)[114], as recently demonstrated in another prototype model of direct injury, Okabe's direct acetic acid application into stomach-induced gastric lesions[115,116], which is also commonly used in cytoprotection studies[1].

Provided that the essential point for lesions in Robert's cytoprotection model would be the injury made by direct contact (damage) to the cells[14-16], the perforation lesion instantly made by surgery is thereby a prototype[1]. The healing of perforated injury by the application of BPC 157 is an important conceptual point[1]. Further consequent evidence includes the healing of skin wounds and other wounds[1,3]. Importantly, proper wound healing includes the achievement of all four major events (vascular constriction, loose platelet plug, fibrin mesh to ensure stability of platelet plug, and dissolution of the clot) that occur in a set order following the loss of vascular integrity [3]. As a result, an agent applied in wound healing, such as stable gastric pentadecapeptide BPC 157, which is shown to be effective in wound healing, should also be effective in bleeding disorders[3].

Together, these consistent beneficial effects clearly indicated a full potential, in addition to the achievement of local protection and therapy of the stomach and gastrointestinal tract[1], toward Robert's point (other organ (epithelia) protection and therapy achieved)[14-16]. Of note, as pointed out, these studies indicated the use of the stress gastric ulcer models as a "cytoprotective" model (*i.e.*, not related to gastric acid secretion)[39]. The significance of the stress gastric ulcer models is fairly described in several reviews[117-121]. Likewise, the connection with the prostaglandin system (and thereby, Robert's cytoprotection) is fully substantiated[31]. For BPC 157, the use of the prolonged restraint stress procedure[1] was important, provided that the use of the restraint stress methodology by gradually modulating/increasing the level of the stress[39,120] (*e.g.*, usual cold + 3 h[39,120] *vs* 48 h restraint stress[1]) fully highlighted its efficacy[1]. Thereby, we could consistently suggest the effectiveness of BPC 157 over the application of standard H2-blockers or dopamine agonists[1].

Likewise, providing protection against the possible negative influence of gastric acid (hyper)secretion, in addition to the counteraction of Shay stomach ulcers induced by pylorus ligation[122] (but no influence on gastric acid secretion[123]) by BPC 157, there is some antagonism of the cysteamine-induced duodenal ulcer[1]. Since the Szabo's study[86], cysteamine-duodenal ulcers are commonly related to gastric acid hypersecretion[124-127]. However, we should consider stress lesions[85] as cytoprotection before Robert's cytoprotection[14-16], and thereby, Selye's "stress view"[85]. The introduction of cysteamine duodenal ulcers in rats will overcome the problems arising from multiple gastric erosions as the most characteristic rat gastrointestinal manifestations of exposure to stress, and would closely mimic human "stress ulcers", which are frequently localized in the duodenum[85]. Selye and Szabo considered the duodenal ulcer potency of various agents, and also emphasized "some relation to nonspecific stress" since cysteamine was the most potent agent of the other agents assessed (acetanilide, allylchloride, acetaminophen, 4,4-diaminodiphenylmethane, proprionitrile, and 3,4-toluendiamine) which were capable of inducing such lesions [85]. Yet, at that time, no mention was made on any influence of dopamine or gastric acid secretion[85]. With such particular "stress" notation to the duodenal lesions[85], initiation goes along with the emergence of the histamine, and the H2 receptor blocker-mediated resolution of peptic ulcers[128]. The subsequent cysteamine report by Szabo in the Lancet revealed the dopamine and gastric acid hypersecretion background, meaning that it became a seminal dopamine paper[86].

Also, this beneficial effect in cysteamine-induced duodenal ulcers[1] combined BPC 157 application with the dopamine system. Szabo provided cysteamine as a dopamine antagonist and its close similarity with the parkinsongenic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (that also induced duodenal ulcers)[129, 130] in support of the theory holding schizophrenia, Parkinson's disease, and ulcer disease as dopamine system failures, and dopamine antagonists (ulcerogenic potential)/dopamine agonists (therapy) in peptic ulcer therapy[131]. Lately, the interaction of BPC 157 with the dopamine system was reviewed[2]; BPC 157 counteracted the effect of neuroleptics (haloperidol), MPTP, and reserpine (*i.e.*,

akinesia, catalepsy, hypothermia, and gastric lesions). Also, BPC 157 counteracted the models resembling positive-like symptoms of schizophrenia[55], and haloperidol-induced catalepsy and gastric ulcers[2]. This effect, as a close interaction with dopamine system functioning, was able to determine an active gut-brain axis or brain-gut axis functioning[2]. It should be noted that BPC 157 also counteracted various encephalopathies and behavioral disturbances, and may therefore represent essential brain-gut and gut-brain axis activities[2]. As an extension of the therapeutic effect, BPC 157 also counteracted the typical and atypical neuroleptic-induced arrhythmias, QTc-interval prolongation[1]. The counteraction of the prolonged QT interval appeared as part of the large therapeutic effect of BPC 157 on the heart disturbances noted in the prevention and reversal of doxorubicin-induced chronic heart failure[1], and the counteraction of various arrhythmias[1], including those induced by venous occlusion [9-11].

Subsequently, again with the 96% alcohol-induced gastric lesion[1], the cytoprotective effect of BPC 157 was closely related to the NO system that should have an essential role in the maintenance of gastrointestinal mucosa integrity, and, more importantly, in endothelial functioning[1,8]. BPC 157 induced NO release from homogenate supernatants of the gastric mucosa from the rat stomach, which is particularly resistant to the NOS blocker N(G)-nitro-L-arginine methylester (L-NAME), and may counteract the NOS substrate L-arginine-induced NO over-release[1]. This particular interaction may be seen in various models and species with the ability of BPC 157 to counteract the adverse effects of L-NAME and L-arginine application[1].

Also, an essential point to remember in the cytoprotective effect of agents is capsaicin-sensitive afferent neurons[45,46], which regulate vascular function in many somatic and visceral tissues, including the regulation of local blood flow in the gastrointestinal tract. Thereby, the important point is that the beneficial effect of BPC 157 in gastric lesions induced by ethanol, restraint stress, or indomethacin was combined with the maintained as well as restored capsaicin-sensitive afferent neurons [1]. Quite recently, this cytoprotective notation was confirmed with the evidence that BPC 157 acts *via* inhibition of the release of enteric serotonin, an increase in the rat and human survival rate of cultured enteric neurons, and the proliferation of cultured enteric glial cells (EGCs)[132]. It was suggested that the inhibition of the release of enteric serotonin may be related to the release of serotonin noted in several brain areas (*i.e.*, nigrostriatum) after the administration of BPC 157[2,132].

Together, these findings clearly indicate a complex involvement of BPC 157 in the practical realization of cytoprotection as a non-gastric acid dependent phenomenon and "direct cell injury to cell – direct cell protection" principle. Furthermore, unlike its ulcerogenic effect ascribed to gastric acid hypersecretion[86], we showed that the application of cysteamine after gastrectomy induced duodenal ulcers in gastrectomized rats, and BPC 157, as well as all standard anti-ulcer agents, may clearly antagonize these cysteamine-induced ulcers in gastrectomized rats[1]. Interestingly, sialoadenectomy abolished the beneficial effect of standard antiulcer agents on cysteamine-induced duodenal ulcers, while BPC 157 was also effective in sialectomized rats[1]. As mentioned above, further evidence showed cysteamine enema-induced ulcerative colitis[1]. Thus, these findings may be used as a full argument that cysteamine-induced ulcer appears as originally suggested (stress ulcer, non-gastric acid-dependent)[5,8] while cytoprotection, as the non-gastric acid-dependent phenomenon and "direct cell injury to cell – direct cell protection" principle, is continuously operating[1].

Epithelial pathway for innate cytoprotection both inside and outside the gastrointestinal tract

The wider range of BPC 157 therapy[1,7] follows the definition of the innate cytoprotective potential in additional circumstances (*i.e.*, other epithelial healing), which has to combine the healing of different tissues, and is thus a pleiotropic beneficial effect[14-16]. Provided that cytoprotection represents a huge range of beneficial effects as the prototype model[1], there was a consistent demonstration of the strong therapeutic effect of BPC 157[1]. As emphasized, it not only occurred in the entire gastrointestinal tract[1,2], but also in various liver lesions, acute pancreatitis, and heart, lung, and kidney disturbances[1]. The consistent beneficial effects that include a considerable number of models may clearly verify the large range of therapeutic effects[1]. For instance, there are therapeutic effects in the liver lesion network against prolonged restraint stress, bile duct and hepatic artery ligation, CCl4 application, chronic alcohol drinking, NSAID over-dose application, insulin over-dose, and bile duct ligation-induced cirrhosis[1]. In particular, the beneficial effects occur against ischemia-reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in

rats. Acute pancreatitis models were represented by bile duct ligation or lower esophageal and pyloric sphincter dysfunction[1]. As already mentioned, heart disturbance counteraction[1] was based on doxorubicin-induced chronic heart failure [57], and the counteraction of various, quite distinctive arrhythmias. This may be clearly seen providing the wide range of noxious events tested (*i.e.*, digitalis, hyperkalemia, bupivacaine, and lidocaine)[1] and venous occlusion procedures applied[9-11]. Likewise, the lung lesion counteraction is based on edema of the interstitium, and substantial dilatation and congestion of the capillaries in the alveolar septum in the lung of rats with venous occlusion syndromes[10,11]. If not corrected, the lung congestion appears as a common outcome (*i.e.*, time-dependent and time-independent features that can be acute respiratory distress syndrome exudative phase features); acute lung injury is a primary component of multiple organ dysfunction syndromes triggered by intestinal ischemia-reperfusion, which results in high mortality and acute lung injury[133,134].

Likewise, as a general follow-up of Robert's cytoprotection, the BPC 157 wound healing studies appear to be well founded[1,3]. As well as gastrointestinal ulcers, consistent evidence includes various skin wounds. In addition to the incisional wound and deep burns and fistula wounds[1,3], there were also diabetic ulcers[102,135] and alkali wounds[105]. These beneficial effects also include the healing of muscle (*i.e.*, the healing of the transected, crushed and denervated muscle), tendons (transected Achilles' tendon and Achilles' tendon detached from the calcaneus), ligaments (transected medial collateral ligament), and bone (alveolar bone loss and radial pseudoarthrosis)[3]. The delivery of BPC 157 was through local (*i.e.*, cream application) and systemic (*i.e.*, intraperitoneally, or intragastrically, or per-orally in drinking water) methods[1,3]. The therapeutic effects of BPC 157 on tendon and muscle healing was also investigated[3,103,104,107]. Moreover, there is a strong practical distinction from the standard angiogenic factors[3]. As pointed out, bFGF, EGF, and VEGF gastrointestinal tract studies demonstrated improved healing[3]. However, most of their corresponding studies on tendon, muscle, and bone injuries provide evidence of their increased presentation along with various procedures used to produce beneficial effects, compared to fewer studies *in vitro*[3]. *In vivo* healing evidence of these standard angiogenic growth factors was limited, commonly to local application. Evidently, providing the use of different carriers with corresponding peptides, there is an obvious attribution problem due to different combinations of peptide + carrier complex. Thereby, for the standard growth factors and use of different carriers, there is inadequate evidence due to diverse healing evidence with diverse carriers and delivery systems[3]. Contrary to this, BPC 157, using the same regimens as gastrointestinal healing studies (always given alone, without carrier), improves tendon, ligament and bone healing, accurately implementing its own angiogenic effect in healing[3]. Important for the particular effect on angiogenesis (particular in consideration of the corneal avascularity as "angiogenic privilege", no formation of corneal neovascularization which is essential for corneal wound healing)[136], later studies also included corneal wound healing and maintained corneal transparency (rescued total debridement of the corneal epithelium and perforating corneal incisions)[3]. The evidence that BPC 157 eye drops successfully close perforating corneal incisions in live rats is consistent with the cytoprotection/endothelial/mucosal protection model[3]. Regardless of its complex function in the corneal endothelium, endothelial maintenance by BPC 157 is also implicated in the healing of corneal ulcers in live rats [3]. Since this model is sensible, we suggest that BPC 157 should have tissue-specific healing effects[3]. Thus, we can envisage a particular healing potential in cytoprotection terms. From the method viewpoint[1,3], all of these lesions are within the scope of Robert's direct cell injury produced by direct contact[14-16].

In addition, there is quite indicative evidence about the simultaneous healing of different tissues. There is healing of various anastomoses (vessel, nerve, and gastrointestinal tract) and of various fistulas (surgically induced by defects and anastomosis creation), both external and internal[1]. Together, these findings showed that this additional extent (*i.e.*, the healing of other epithelia) may be combined in the simultaneous healing of different tissues, such as the simultaneous healing of fistula defects and the closing of fistulas[1]. A particular point is that these rat fistulas are severe, considering the significant size of the defect relative to the small size of the corresponding rat tissue[1]. Illustratively, rectovaginal fistulas in rats, with a 5 mm defect *vs* a 2.4 cm vaginal length, result in long-lasting defects and spontaneous patency of the fistula, leading to fecal matter leaking through the vagina; this actually mimics severe fistulas that may not spontaneously heal, thereby clearly emphasizing the beneficial effect of BPC 157[1].

Further, for BPC 157, in an additional cytoprotective extent (*i.e.*, other epithelia healing), epithelium protection is based on the extended relevance of the intragastric alcohol or NSAIDs on the stomach lesions commonly used in Robert's cytoprotection studies[1,14-16]. Namely, Robert's first epithelium protection, or the direct cell protection against cell injury produced by direct contact with the noxious agents, used intragastric alcohol or NSAIDs to induce stomach lesions[14-16]. Consequently, further evidence toward an additional extent (*i.e.*, other epithelia healing) follows other adverse effects of alcohol and NSAIDs and their consistent counteraction[1,7].

In addition to the 96% alcohol intragastric application-induced gastric lesions, BPC 157 largely counteracted chronic alcohol drinking-induced stomach lesions, liver failure, and portal hypertension, providing evidence that it may act as an alcohol antagonist[1]. Likewise, BPC 157 promptly counteracted acute alcohol (4 g/kg intraperitoneally) intoxication (*i.e.*, quickly produced and sustained anesthesia, hypothermia, increased ethanol blood values, 25% fatality, 90-min assessment period) given before or after ethanol[1]. In addition, BPC 157 counteracted chronic (withdrawal) alcohol intoxication, and was suggested as an alcohol antagonist[1], peripherally and centrally (of note, BPC 157 may attenuate the effect of thiopental anesthesia)[2].

Confronted with the over-dose application of various NSAIDs[1], similar beneficial effects occurred against various gastrointestinal lesions, and liver and encephalopathies; the worst damaged areas showed the most evident therapeutic effect[1]. Prolonged bleeding, consequent thrombocytopenia, and thrombocyte malfunctioning were also attenuated and/or counteracted[1,3,7]. Therefore, it seems that BPC 157 may particularly affect the functioning of the prostaglandins system[1,3,7] (interestingly, unlike NSAIDs and corticosteroids, BPC 157 strongly prevented adjuvant arthritis development and reversed the already formed adjuvant arthritis in rats[1]). The final clue may be that BPC 157 counteracted indomethacin-induced leaky gut syndrome[6]. It acts *via* increasing tight junction protein ZO-1 expression, and transepithelial resistance, inhibiting the mRNA of inflammatory mediators (*iNOS, IL-6, IFNγ*, and *TNF-α*), and increasing the expression of HSP 70 and 90, and antioxidant proteins, such as HO-1,NQO-1, glutathione reductase, glutathione peroxidase 2, and GST-pi[6]. Considering the importance of the leaky gut as an essential mechanism responsible for various severe systemic diseases, this may fully substantiate the significance of BPC 157 in the realization of that additional cytoprotective extent (*i.e.*, other epithelia healing)[6]. Also, BPC 157 counteracted other encephalopathies induced by various noxious events (insulin over-dose, cuprizone, multiple sclerosis mimicking neurotoxin, magnesium over-dose, brain trauma, spinal cord compression, and stroke)[1,2].

Epithelial pathway for adaptive cytoprotection

We also demonstrated that BPC 157 may regulate cytoprotection adaptation processes (adaptive cytoprotection)[1], functioning of the endogenous adaptive processes essential for permanent mucosal maintenance, and afford defensive reactions that start after any injurious event[1]. This follows Robert's connotation about the cytoprotection as a physiologic process[14-16] based on the adaptive cytoprotection evidence of Robert's small irritant to the stomach that precedes and protects against any subsequent major injurious event (*i.e.*, Robert's strong irritant to the stomach)[17]. Evidently, cytoprotective agents should have a more extensive action, participate in Robert's first epithelium protection, exhibit direct cell protection against cell injury produced by direct contact with noxious agents, and also participate in adaptive cytoprotection, in the next defensive reaction, and afford its final beneficial effects (*i.e.*, permanently attenuated lesion consequences)[1]. Thus, whatever the small irritant may be, whether exogenous (mild alcohol) or endogenous (*i.e.*, accumulated gastric juice, gastric acid, *i.e.*, made by gastrojejunal anastomosis), BPC 157 administration strongly contributed to the final attenuation of stomach lesions[1]. Thus, BPC 157 strongly contributes to and improves the presentation of adaptive cytoprotection processes[1]. Specifically, BPC 157 would improve adaptation processes in the damaged intestine, through a prostaglandin-related process, as it may be strongly aggravated by the application of NSAIDs[1]. In rats with short bowel surgery, the BPC 157 therapy, per-oral (in drinking water) and parenteral, causes constant weight gain (even more than preoperative values), with all three wall layers accordingly increased (*i.e.*, villus height, crypt depth, and muscle thickness [inner (circular) muscular layer] also increased), but no difference in jejunal and ileal diameters, and increased anastomosis strength. These beneficial effects of BPC 157 (*i.e.*, the weight gain in the BPC 157 rats with short bowel, all three wall layers accordingly increased) appear to be particular[1]. Namely, standard growth factors [even using a special application route (*e.g.*, subcutaneous pump)][137,138] at best may induce a decrease in weight loss[139-

142], with an increase in one layer, but not in the other. There is also some caution about the use of peptidergic agents, and adaptation processes, particularly on a long-term basis[140]. There is some growth of several tumor cell lines (EGF)[143,144], and hyperplastic lesions in the colon (subjects treated with GLP-2[145]). In contrast to adequately controlled adaptive processes, supportive evidence for BPC 157 (*i.e.*, BPC 157 administration showed no toxic effect and was limit test negative, with LD1 not achieved, and no side-effects in trials[1,3]) shows that it inhibits the growth of several tumor cell lines and counteracts the tumor-promoting effect of vascular endothelial growth factor (VEGF)[1,4]. In mice with C26 colon adenocarcinoma, BPC 157 counteracted tumor-cachexia and markedly prolonged survival[5]. BPC 157 afforded significant mitigating action against cancer cachexia-induced muscle degeneration, inflammation, and catabolism. BPC 157 significantly corrected deranged muscle proliferation as well as myogenesis, counteracted an increase in proinflammatory cytokines such as IL-6 and TNF-α looking at muscle metabolism relevant to cancer cachexia, as well as any changes in the expression of FoxO3a, p-AKT, p-mTOR, and P-GSK-3β[5]. Also important for its likely control of the adaptation processes, and prostaglandins-system function, in the short bowel rats, BPC 157 may counteract gastrointestinal lesions and the concomitant liver and brain lesions, and the additional aggravation that would otherwise appear with the application of diclofenac[1].

Also, it is possible that BPC 157 would afford an adaptive cytoprotective reaction regardless of the site of its initiation in the gastrointestinal tract[1]. Supporting evidence was also provided showing that adaptation cytoprotection accordingly occurs in the complete gastrointestinal tract, lasting for a considerable time, depending on the part that is initially targeted by the small irritant, stomach, duodenum, or colon, enabling the other parts to be more resistant to any subsequent strong irritant challenge[1]. Considering the eating and drinking habits, the adaptive cytoprotection in the gastrointestinal tract starts in the upper parts, in the stomach and duodenum, and may beneficially affect other parts (and thereby, adaptive cytoprotection occurs between stomach → stomach; stomach → duodenum, stomach → colon; duodenum → duodenum; duodenum → stomach, duodenum → colon)[1]. The colon seems to be distinctive and passive, as it could not initiate an adaptive cytoprotection response[1]. We used combinations of specific agents for initial small lesion and final more severe lesion [1 mL/rat of 25% or 96% ethanol intragastrically (stomach); cysteamine 40 mg/kg or 400 mg/kg subcutaneously (duodenum); cysteamine 40 mg/kg or 400 mg/kg intrarectally (colon)][1]. All of these ulcerogens were known to be inhibited by BPC 157[1].

Finally, with normal eating and drinking, Robert's adaptive cytoprotection (*i.e.*, Robert's small irritant to the stomach and Robert's strong irritant to the stomach) showed another essential point. We used the tongue as the initial target[1]. Within the very short time needed to swallow, the stomach is immediately affected, and the lesions are considerably less than those obtained with the direct instillation of alcohol into the stomach[1]. The application of BPC 157 considerably afforded this spontaneous healing effect, and additionally mitigated tongue, esophageal, gastric, and duodenal lesions, and reversed lower esophageal and pyloric sphincter impairment, through an action which seems to be NO system dependent[1]. Actually, it means that Robert's cytoprotection and adaptive cytoprotection following the direct application of noxious agents into the stomach completely avoid the regular defensive response that would occur with the tongue (and not the stomach) as the initial target.

On the other hand, this emphasizes the original significance of Robert's application of alcohol directly into the stomach, and thereby cytoprotection, as the direct cell protection against direct cell injury produced by direct contact with the noxious agents. Robert's regimen (alcohol applied in the stomach directly, by tube) regularly skips the existing defensive system (*i.e.*, starting with the tongue). Consequently, spontaneous rapid healing mechanisms remain skipped and not activated. Thereby, the essential ability of the cytoprotective agents would depend more on their own healing capacity, and their ability to act rapidly to induce healing.

Thus, such a huge range of healing effects, as noted with the applications of BPC 157, should be a prerequisite for realizing the endothelium pathway (blood vessel recruitment and activation towards defect or bypassing vessel occlusion)[1,7].

Endothelium pathway (endothelium maintenance to epithelium maintenance)

We already emphasized[1,7] the original cytoprotection studies[14-16,20,21] from the 1980s, which demonstrated significant stomach endothelium lesions, and verified the consequent change in stomach injuries *via* the endothelium pathway (endothelium maintenance → epithelium maintenance)[14-16,20,21]. Since that time, the cytoprotective endothelium pathway remained to be fully elaborated for therapeutic

purposes, both as a stomach therapy and as a more general therapy. With this high therapy requirement, we summarize the additional evidence. These highlights include the Virchow triad situation, endothelium injury, thrombus, and stasis, preceding the current demonstration that the administration of BPC 157 may finally induce the rapid recruitment of existing blood vessels and activate particular collateral pathways when confronted with vessel occlusion[1,7,9-11]. That pathway activation would accordingly compensate for the occlusion of vessels, and mitigate the consequent noxious chain of events[1,7,9-11]. We would analyze the possible cause in the indicated cytoprotection terms leading to an extension of the endothelium pathway to blood vessel recruitment and activation towards defect or bypassing vessel occlusion[1,7,9-11].

After presenting the initial cytoprotection concept (*i.e.*, epithelial pathway)[14-16], the endothelial pathway appeared as a further clarification of the development of stomach lesions in the concept of cytoprotection[14-16,20,21]. As a common point[47] appeared the evidence that with alcohol intragastric instillation, these vascular changes are early events, even before the appearance of gross hemorrhagic lesions, occurring within seconds or minutes during the development of moderate or severe gastric mucosal injury with interstitial hemorrhage and the necrosis of glandular epithelial cells[14-16,20,21]. Additionally, there is early stasis of mucosal blood flow and thrombi formation (within 30 s), often in regions without deep necrotic lesions. Even more, there was the rapid and complete cessation of blood flow to areas of mucosal damage consequent to ethanol administration[146]. Thus, although this was not initially claimed for the beneficial effect of prostaglandin and cysteamine application[14-16], we can envisage the particular Virchow triad presentation[1,7]. Unlike the initial claim for generalization of the epithelial stomach protection to other epithelial protection (cytoprotection → organoprotection)[14-16,19], at that time, these studies[14-16,20,21] made no attempt to generalize the findings seen for endothelium recovery in the stomach.

A strong reduction of Monastral blue staining and maintenance of the endothelium integrity after alcohol intragastric application was considered to be essential for the healing effect of BPC 157[1]. An interesting insight appeared after absolute alcohol instillation in the fully distended rat stomach, and gastric, esophageal, and duodenal lesions. Throughout the next 3 min, left gastric artery blood vessels clearly disappeared at the serosal site, indicative of the loss of vessel integrity and function. In contrast, constant vessel presentation could predict the beneficial effect of the applied agent. After pentadecapeptide BPC 157 instillation into the stomach, the vessel presentation remained constant, and lesions of the stomach, esophagus, and duodenum were inhibited[1]. Standards (atropine, ranitidine, and omeprazole) could only slightly improve the vessel presentation compared to control values, and only had a partial effect on the lesions[1]. Furthermore, for BPC 157, this maintenance of the endothelium integrity initially revealed a strong inhering angiogenic effect, which was more potent than those noted for standard antiulcer agents[1,7]. This appeared as a follow-up of "direct" cellular pharmacological treatment for ulcer with growth factors, notably bFGF and PDGF, that should result in the superior quality of ulcer healing by optimal angiogenesis, and thereby dense granulation tissue, as well as the complete re-epithelization and restoration with minimal inflammation[32]. Moreover, with BPC 157, it appears that angiogenesis was closely related to its wound healing and promotion, as well as healing in other tissues (*i.e.*, muscle, tendon, and ligament, known to be hypovascular tissues)[3]. In particular, in both muscle (transected or crushed) and transected tendon healing, we noted an increase in early angiogenesis (and the increased expression of VEGF, Factor VIII, and CD34), while late angiogenesis decreased (and the expression of VEGF, Factor VIII, and CD34 was decreased)[3]. The therapeutic potential (*i.e.*, acceleration of the blood flow recovery and vessel number in rats with hind limb ischemia) of pro-angiogenic BPC 157 is associated with VEGFR2 activation and up-regulation[107]. It also immediately triggered the internalization of VEGFR2 and subsequently activated the phosphorylation of VEGFR2 and Akt, and the eNOS signaling pathway without the need for other known ligands or shear stress [107].

On the other hand, as the reduction of Monastral blue staining and maintenance of the endothelium integrity after alcohol intragastric application is an immediate effect of BPC 157, we should consider the pleiotropic beneficial effects of BPC 157 in the entire gastrointestinal tract[1,7]. This should provide evidence that it effectively combines its particular mediator role (as an original anti-ulcer peptide which is stable in human gastric juice for longer than 24 h[12]) and thereby, in Robert's stomach cytoprotection, protection against direct cell injury made by direct contact of various noxious agents and required endothelium protection and maintenance of the endothelium function[1,7]. This has to be an immediate and rapid effect[1,7]. Thereby,

BPC 157, as a cytoprotective agent in the entire gastrointestinal tract, may both prevent and reverse the Virchow triad situation, and have an additional modulatory role[1,7].

As the first evidence of the implementation of the endothelium maintenance originally noted in stomach cytoprotection studies[1], BPC 157 prevents and reverses thrombosis formation after abdominal aorta anastomosis, or major vein occlusion[1,7,9-11]. Furthermore, BPC 157 may attenuate the prolonged bleeding that appeared after different injuries (*i.e.*, tail or leg amputation, organ perforation, and prolonged occlusion of the inferior caval vein) or anticoagulants, such as heparin or warfarin, and aspirin and the NOS substrate L-arginine application[1,7,9]. Also, it was shown that BPC 157 maintains thrombocyte function, without interfering with coagulation pathways[1,7]. Furthermore, there is evidence that BPC 157 counteracted stroke, given in reperfusion, after clamping of the common carotid arteries [*i.e.*, both early and delayed neural hippocampal damage, achieving full functional recovery (Morris water maze test, inclined beam-walking test, and lateral push test)][147]. Together, this may be a particular modulatory effect or NO-system-related effect[1]. BPC 157 may counteract both the NOS blocker L-NAME's pro-thrombotic effect and the NOS substrate L-arginine's anti-thrombotic effect in the same way that it counteracted both L-NAME-induced hypertension and L-arginine-induced hypotension, and could induce the NO release on its own, which is quite resistant to L-NAME application[1]. Finally, in addition to the VEGFR2-Akt-eNOS signaling pathway being activated without the need for other known ligands or shear stress[107], there is a direct effect on vasomotor tone (*i.e.*, specific activation of Src-Caveolin-1-endothelial nitric oxide synthase (eNOS) pathway)[106]. Also, it should be recalled that four major events (vascular constriction, loose platelet plug, fibrin mesh to insure stability of platelet plug, and dissolution of the clot) are implicated in the wound healing process and occur in a set order following the loss of vascular integrity[3]. Consequently, it may be not surprising that an agent implemented in wound healing, such as stable gastric pentadecapeptide BPC 157, should be effective in this particular way also in bleeding disorders[1,3], due to its innate cytoprotective effect, and the fact that it has been shown to be an effective therapy in wound healing[3].

Finally, in consideration of the previous original findings in cytoprotection endothelium studies (complete cessation of blood flow to areas of mucosal damage and rapid cloth formation consequent to ethanol administration[146]), and resolving of the presented Virchow triad circumstances, we suggested that the beneficial effect of cytoprotective agents should be related to the resolution of this noxious chain of events[1,7]. Thus, conclusive evidence involves confrontation with permanent major vessel occlusion, and therapeutic evidence that BPC 157 administration quickly recruits vessels to rapidly activate the collateral pathway which would adequately compensate for vessel occlusion and reestablish blood flow[1,7,9-11]. There, the alleviated peripheral vascular occlusion disturbances rapidly activated alternative bypassing pathways[1,7,9-11], appears to be an additional follow-up of its essential endothelium protection[1], which was long ago implemented as an essential class activity of cytoprotective agents[13]; however, in this way, this has so far only been implemented by the application and beneficial effects of the stable gastric pentadecapeptide BPC 157[1,7,9-11].

Rapid activation of a bypassing loop from the existing vessels

With BPC 157 therapy, when confronted with the occluded vessel in rats with distinctive vascular occlusion disturbances, we first reported the rapid activation of a bypassing loop recruited from the existing vessels (*i.e.*, intestinal arcade vessel network, or the left ovarian vein)[1,7,9-11]. The evidence [1,7,9-11] included the infrarenal occlusion of the inferior caval vein, left colic artery and vein occlusion ischemic/reperfusion ulcerative colitis, superior anterior pancreaticoduodenal vein-induced duodenal venous congestion lesions, bile duct ligation-induced liver cirrhosis and portal hypertension, temporary occlusion of the portal triad (Pringle maneuver)-induced ischemia-reperfusion injury[10], and suprahepatic occlusion of the inferior caval vein-induced Budd-Chiari syndrome[11]. This occurred in rats with a ligated part of the left colic artery and vein, ischemic/reperfusion colitis, or an infrarenal ligation of the inferior vena cava[1,7,9-11]. Evidently, the BPC 157 application-induced activation of the collateral pathways (the left ovarian vein and other veins in rats with infrarenal occlusion of the inferior caval vein) may rapidly resolve any systemic disturbances (*i.e.*, caval hypertension, aortal hypotension, heart dysfunction, thrombosis, and consequent thrombocytopenia, and induced bleeding prolongation in rats with infrarenal occlusion of the inferior caval vein)[1,7,9-11]. Likewise, there is also the local injury counteraction (attenuated/counteracted ischemia/reperfusion injury) in a rat study of the ischemic/reperfusion colitis[1,7]. As emphasized[1], with

part of the left colic vein and artery excluded by two ligations, along with BPC 157 application, blood vessels propagated toward the injury obstruction, bypassing it, interconnecting collaterals between arcades, and reestablishing the inside-outside point. In reperfusion, the application of BPC 157 after the initiation of full reperfusion with both ligations removed resulted in increased vessel presentation and arcade interconnections. With application of BPC 157 in ischemia as well as in reperfusion, the mucosal folds were recovered, and the pale areas were small and markedly reduced in size[1]. In the ischemia and even more so in the reperfusion, oxidative stress was counteracted, and the otherwise increased MDA (as a result of the lysis of endothelial cells[148,149]) and NO levels in colon tissue were found to be normal in rats that received BPC 157 bath treatment[1]. This occurs as before in both ischemic and reperfusion conditions in the various tissues (*i.e.*, the colon, duodenum, cecum, liver, and veins) and plasma[1,7,9-11]. Thus, the action of BPC 157 as a free radical scavenger (noted also in the other tissues, *i.e.*, gastrointestinal sphincters, stomach, duodenum, bowel adhesions, bladder, and brain[1,7,9-11]) may considerably contribute to its pleiotropic beneficial effects and maintain endothelial function. Notably, BPC 157 contains four carboxylic groups that could be active in scavenger process, and if they are reactivated (by, *e.g.*, glutathione or enzymes), the overall antioxidant activity could be very high [1].

Thus, relieving Virchow's triad situation is the particular activation of collateral pathways corresponding to the damaging occlusion [*i.e.*, mentioned passing through arcade vessels (occlusion of the left colic artery and vein) or the left ovarian vein (infrarenal occlusion of the inferior caval vein)][1,7,9-11]. As pointed out[1,7,9-11], the superior anterior pancreaticoduodenal vein-inferior anterior pancreaticoduodenal vein-superior mesenteric vein appears to counteract duodenal congestion lesions[1,7,9-11]. A porto-caval shunt appears with the portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway to counteract portal hypertension in rats with bile duct occlusion or ischemia-reperfusion injury following Pringle maneuver[1,7,9-11]. The inferior caval vein - azygos vein - left superior caval vein pathway appears to counteract Budd-Chiari syndrome in rats[1,7,9-11].

Of note, an adequate compensation regularly occurred. As pointed out in our venous occlusion studies[10,11], there is consistent evidence in rats with bile duct ligation. Preventing the development of portal hypertension, and the rapid reversal of the already established portal hypertension, are both among its additional beneficial effects[150]. We noted that BPC 157 therapy markedly abated jaundice, ascites, and nodular, steatotic livers with large dilatation of the main bile duct, increased liver and/or cyst weight, and decreased body weight[150]. Furthermore, the piecemeal necrosis, focal lytic necrosis, apoptosis, and focal inflammation, disturbed cell proliferation (Ki-67-staining), cytoskeletal structure in the hepatic stellate cell (α-SMA staining), and collagen presentation (Mallory staining) were all counteracted, providing evidence that BPC 157 may affect both liver fibrosis and portal hypertension [150]. Thus, this may be the principle seen in venous occlusion studies[10,11].

As previously reviewed[1], in rats with a perforated cecum, BPC 157 application rapidly reversed the regular noxious course, with the rapid disappearance of blood vessels at the cecum serosa (emptied/disappeared), thereby producing a large immediate defect, with bleeding, the leakage of fluid, increased oxidative stress, and disturbed NO-levels in cecal tissue. With BPC 157, there is immediate blood vessel recruitment and activation ("running") towards the site of injury[150], as was described in the "bypassing" of vessel occlusion *via* alternative pathways[9-11], which can likely cure rats and reestablish blood flow. Also, a small-vessel network appeared around the perforated defect with BPC 157 bath administration; cecal defect enlargement reversed to defect contraction (*i.e.*, each defect breaks blood flow) may be a result of the reestablishment of blood flow as well as the shortened bleeding time from the perforated cecum[1]. Less bleeding corresponds to the beneficial effects in rats with amputation, anticoagulant or aspirin application, or vein obstruction; direct defect closing corresponds to the closing of various fistula defects, which were also surgically created in corresponding tissues[1,7,9] (*i.e.*, all by Robert's direct injury to the cell by direct contact).

Along with these findings[1,7,9-11] is the beneficial effect of BPC 157 in rats with a damaged peritoneum. Endothelium maintenance → epithelium maintenance = blood vessel recruitment and activation ("running") towards the site of injury, also described as "bypassing" the occlusion *via* alternative ways[1,7,9-11], was seen with BPC 157 administration after parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats to counteract failed vasculature, and finally to counteract the increased formation of adhesions. Rapid abundant vascular vessels in and close to the

defect mean that BPC 157 could interfere with the motion of the coagulation cascade once the peritoneum is damaged[1,7,9-11]. When two damaged peritoneal surfaces come into contact with each other, BPC 157 is likely to interfere the temporary role of fibrin in healing without adhesions that must be degraded by the fibrinolytic system for the restoration of normal tissue structure and function, as it reversed the healing that would result in fusion to form a connection, *e.g.*, an adhesion[151,152].

Finally, with the BPC 157 therapy in the Pringle maneuver in rats[10], severe preportal hypertension, temporary portal triad obstruction, ischemia, and short and prolonged reperfusion, we resolved the regular lack of adequate portocaval shunting as the most detrimental feature that should be counteracted[10]. With the stable gastric pentadecapeptide BPC 157, we noted the resolution of damage, either following occlusion or following the re-opening of the hepatic artery, portal vein, and bile duct. Therefore, in the portal triad obstruction syndrome in rats, in the rapidly activated manner, portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway would appear as specific activation of the collateral circulation, as the bypassing loop that can rapidly circumvent occlusions and decompress portal triad obstruction in rats upon BPC 157 administration[10]. That solution in rats with ischemia and reperfusion following the Pringle maneuver goes along with the resolution of oxidative stress, hemodynamic disturbances, severe portal and caval hypertension, aortic hypotension, rapid cloth formation in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, and hepatic artery, ascites, peaked P waves, tachycardia, increased serum values, and gross intestine, liver, lung, spleen, and heart lesions[19]. In particular, it goes along with the application of agents during reperfusion. Furthermore, the pentadecapeptide BPC 157 resolved the suprahepatic occlusion of the inferior caval vein in a Budd-Chiari syndrome model in rats[11]. Budd-Chiari syndrome was perceived as originally suggested[153,154], a hepatic venous outflow obstruction and its manifestation, regardless of cause, but this was mostly attributed to thrombosis, which can be located anywhere from the small hepatic veins to the entrance of the inferior vena cava into the right atrium[153,154]. Thereby, bypassing the occlusion in the rat Budd-Chiari syndrome along with pharmacotherapy treatment should be essential. BPC 157 therapy results in the rapidly activated azygos/hemiazygos vein bypassing pathway, upgrading an inadequate rescuing inferior-superior vena cava shunt to an adequate one, as well as a portocaval shunt[11]. Consequently, the caval and portal hypertension and aortal hypotension presented by Budd-Chiari syndrome rats were largely eliminated by BPC 157 therapy [11]. Largely attenuated consequent disturbances (rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST elevation, tachycardia, gross organ lesions, and liver and spleen weight increases) together support this contention [11].

Thus, BPC 157 application may counteract a life-threatening syndrome[9-11]. Characterized by the multiple mutual cause-consequence relationships in vascular occlusion-induced syndrome presentation in rats, the generalized thrombosis and stasis, vascular failure, and heart dysfunction, lung congestion appears to be a common outcome (*i.e.*, time-dependent and time-independent features that the exudative phase features of acute respiratory distress syndrome)[9-11]. Acute lung injury is a primary component of multiple organ dysfunction syndrome triggered by intestinal ischemia-reperfusion. The results may be high mortality and acute lung injury[155,156], followed by liver failure (substantial congestion of central vein as well as branches of portal veins in portal triads), kidney congestion, prominent portal and caval hypertension, aortal hypotension, and consequential gastrointestinal hemorrhagic lesions[9-11]. Therefore, the previously mentioned beneficial effects, in elaborating the cytoprotective "epithelial pathway" (*i.e.*, counteracted various heart or liver lesions), including the combined and simultaneous healing of different tissues[1], may also be essential. In particular, the compensatory efficacy of new functional equilibrium ("endothelium pathway") with the activated specific functioning collateral pathways[13-15] is also ascertained with an important notification for the general pathology of the portal hypertension[1,7]. Namely, BPC 157 counteracted all portal hypertension presentations whatever the cause, post-hepatic, hepatic, and pre-hepatic [1,9-11].

In addition, as in venous-occlusion syndromes[9-11], BPC 157 also counteracted various lung lesions[1,7].

Finally, with holistic concepts, any criticisms about the cytoprotection concept, such as "cytoprotection", "as everything and nothing", and "cytoprotection which is not mechanism", and thereby, criticisms about peptides and cytoprotection, could not be avoided. The general point that animal studies *per se* may be cautious regarding their

results and the relative paucity of BPC 157 clinical data was also reported[1]. On the other hand, it should be noted that BPC 157 was proven to be efficacious in ulcerative colitis, both in clinical settings[157,158] and in experimental ischemic/reperfusion ulcerative colitis studies in rats and other ulcerative colitis models[1]. A particular point is the very safe profile (LD1 could be not achieved)[1,3,7], a point that was recently confirmed in a large study by Xu *et al*[159]. In this context, the role of the animal model is indispensable, and the practical evidence is even more important. Besides the majority of studies with BPC 157 conducted on rodents that were given an injection of the supplement, there have also been a considerable number of studies, particularly in gastrointestinal research, with intragastric application or peroral application in drinking water (regularly used in fistulas studies[1,3]), that are correspondingly effective. There are also studies in other species, *i.e.*, birds and insects (given in the food), which favor a more general effect of BPC 157 application[1,7]. Lastly, the suitability of the models used for the topic of cytoprotection, in particular, since Robert's original description of the cytoprotection in rats[14-16], evidently resolves the practical/theoretical consideration of the cause-consequence issue. Thus, the suited models and lesion counteraction clearly indicate the beneficial effects. The deciding result exemplified the resolved endothelium pathway (blood vessel recruitment and activation towards defect or bypassing vessel occlusion)[1], but the particular background still needs to be further elaborated. Note, the consistently used range of BPC 157 application (μg-ng) may also suggest a physiological role, in accordance with *in situ* hybridization and immunostaining for BPC 157 in the gastrointestinal mucosa, lung bronchial epithelium, epidermal layer of the skin, and kidney glomeruli[3]. Thereby, illustrative examples for further research may be the evidence that BPC 157 exhibited a specific effect on the Egr, Nos, Srf, Vegfr, Akt1, Plcγ, and Kras pathways in infrarenal occlusion-induced inferior caval vein syndrome in rats. This appears in a timely manner, to be increased, decreased, or unchanged, depending on whether the vessel was blinded (the right ovarian vein and inferior caval vein) or open and served as an alternative operating pathway (the left ovarian vein)[9]. Also, to support the beneficial effect of BPC 157 on brain lesions, given in reperfusion in stroke rats[147], BPC 157 therapy counteracted both early and delayed neural hippocampal damage, showing that achieving full functional recovery can restore recognition memory deficits along with a therapeutic effect[147]. mRNA expression studies at 1 h and 24 h, provided strongly elevated (*Egr1, Akt1, Kras, Src, Foxo, Srf, Vegfr2, Nos3,* and *Nos1*) and decreased (*Nos2* and *Nfkb*) gene expression (*Mapk1* not changed). This may be how BPC 157 acts[147].

In conclusion, Robert's cytoprotection concept[14-16] was initially of intense interest, but lately received the claim that the concept's foundation ("gastric cytoprotection") is still relevant[23]. Anyway, the essential rebuilding was lacking. Now, the concept has been reexamined for many major reasons (Figure 1): (1) The gastric pentadecapeptide BPC 157, thought to be an essential cytoprotective mediator that is native to and stable in human gastric juice, was noted to have a pleiotropic beneficial effect[1,3]; (2) With the administration of BPC 157, in prophylactic as well as in therapeutic regimens, there is evidence of the innate Robert's cell protection in the stomach epithelium against direct injury (which may be induced by various noxious agents) using either method of application, which provides the ability to realize the protection of other epithelia as well[1-3]; (3) BPC 157 effectively combines its particular mediator role (as an original anti-ulcer peptide that is stable in human gastric juice for longer than 24 h); therefore, in Robert's stomach cytoprotection, it has a protective effect against direct cell injury made by the direct contact of various noxious agents, requiring endothelial protection and the maintenance of endothelial function. This has to be an immediate and rapid effect[1]; (4) As first evidence of the implementation of the endothelial maintenance originally noted in stomach cytoprotection studies, BPC 157 prevents and reverses thrombosis after abdominal aorta anastomosis, or major vein occlusion[1,7,9-11]. Furthermore, BPC 157 may attenuate the prolonged bleeding that appeared after different injuries or anticoagulant, heparin or warfarin, and aspirin application[1,7,9-11]. Also, BPC 157 maintains thrombocyte function, without interfering with coagulation pathways[1,7,9-11]; and (5) The vessel recruitment activated collateral pathways to bypass vessel occlusion as a new conceptual point in the cytoprotection concept, and cytoprotective agent activity[1,7,9-11]. BPC 157 counteracted various venous occlusion-induced syndromes, inferior caval vein syndrome[9], ischemia/reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in rats. Activation of the alternative collateral pathways to bypass occlusion, and reestablishing alternative blood flow result in counteraction of the consequent syndromes[1,7,9-11]. Due to the severe venous occlusion-induced disturbances, the high portal and caval hypertension and aortal hypotension, arterial

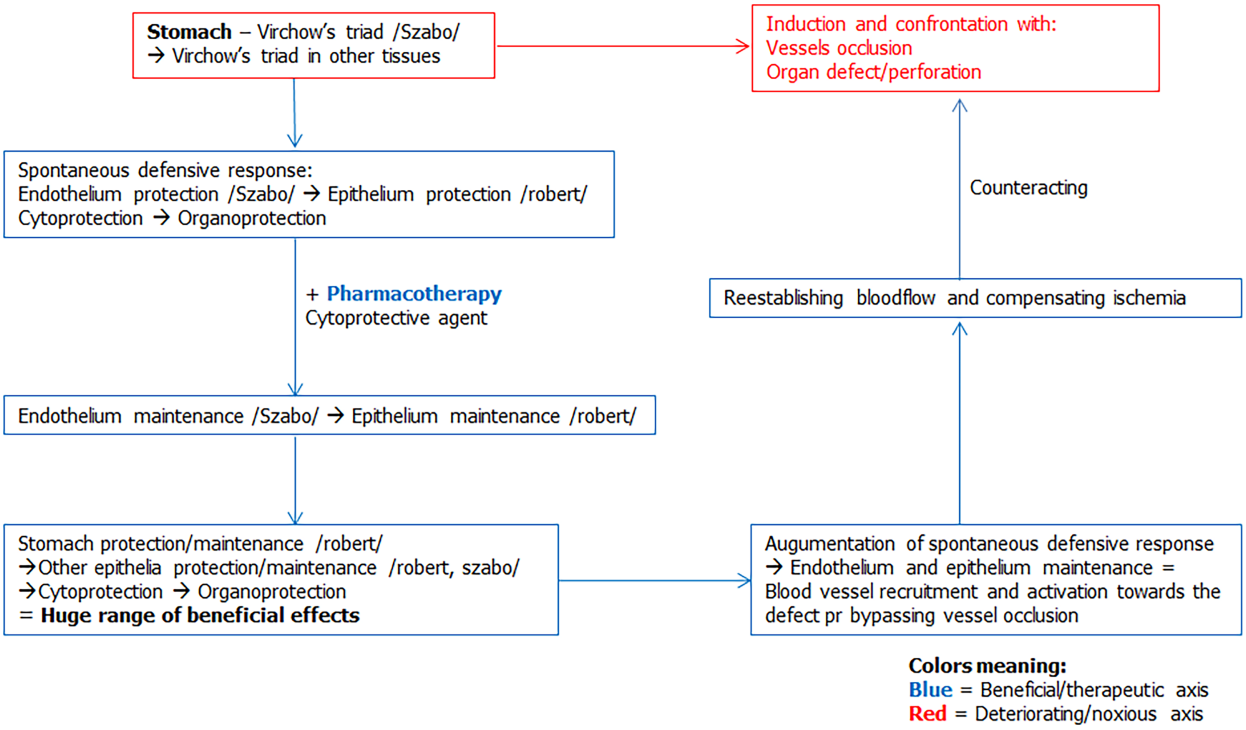


Figure 1 Summarizing the essential epithelium and endothelium protection interplay known in Robert's and Szabo's cytoprotection concept, and the role of the stable pentadecapeptide BPC 157 as a likely mediator, we suggest that BPC 157 may be a useful cytoprotective therapy. Hopefully, it may finally realize in the practice the huge theoretical importance of all aspects of the cytoprotection concept. Conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance (the recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). BPC 157 counteracts various venous occlusion-induced syndromes, inferior caval vein syndrome, ischemia-reperfusion injury following the Pringle maneuver, and Budd-Chiari syndrome in rats. Activation of the alternative collateral pathways to bypass occlusion, and reestablishing alternative blood flow, result in the counteraction of the full consequent perilous syndromes.

and venous thrombosis, both peripherally and centrally, and various organs lesions (*i.e.*, gastrointestinal, liver, kidney, heart, and brain) were all attenuated and/or eliminated[1,7,9-11]. Furthermore, this particular beneficial effect may be competing with the Virchow's triad situation that is commonly presented [*i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, bile duct ligation-induced liver cirrhosis and portal hypertension, portal triad temporary occlusion (ischemia-reperfusion injury following the Pringle maneuver), and suprahepatic occlusion of the inferior caval vein (Budd-Chiari-syndrome)][1,7,9-11,150].

CONCLUSION

BPC 157 may be a useful cytoprotective therapy, which may finally result in the huge theoretical to practical importance of all aspects of the cytoprotection concept[1,7,9-11].

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REVIEW

Transfusion-transmitted hepatitis E: What we know so far?

Carmen Ka Man Cheung, Sunny Hei Wong, Alvin Wing Hin Law, Man Fai Law

ORCID number: Carmen Ka Man Cheung 0000-0001-9386-506X; Sunny Hei Wong 0000-0002-3354-9310; Alvin Wing Hin Law 0000-0001-8376-4193; Man Fai Law 0000-0003-2462-6625..

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Carmen Ka Man Cheung, Man Fai Law, Medicine and Therapeutics, Prince of Wales Hospital, Hong Kong 852, China

Sunny Hei Wong, Institute of Digestive Disease and Department of Medicine and Therapeutics, the Chinese University of Hong Kong, Hong Kong 852, China

Sunny Hei Wong, Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 639798, Singapore

Alvin Wing Hin Law, West Island School, Hong Kong 852, China

Corresponding author: Man Fai Law, MRCP, Doctor, Medicine and Therapeutics, Prince of Wales Hospital, 30-32 Ngai Shing Street, Shatin, Hong Kong 852, China.
mflaw99@yahoo.com.hk

Abstract

Hepatitis E virus (HEV) is a major cause of viral hepatitis globally. There is growing concern about transfusion-transmitted HEV (TT-HEV) as an emerging global health problem. HEV can potentially result in chronic infection in immunocompromised patients, leading to a higher risk of liver cirrhosis and even death. Between 0.0013% and 0.281% of asymptomatic blood donors around the world have HEV viremia, and 0.27% to 60.5% have anti-HEV immunoglobulin G. HEV is infectious even at very low blood concentrations of the virus. Immunosuppressed patients who develop persistent hepatitis E infection should have their immunosuppressant regimen reduced; ribavirin may be considered as treatment. Pegylated interferon can be considered in those who are refractory or intolerant to ribavirin. Sofosbuvir, a nucleotide analog, showed modest antiviral activity in some clinical studies but sustained viral response was not achieved. Therefore, rescue treatment remains an unmet need. The need for HEV screening of all blood donations remains controversial. Universal screening has been adopted in some countries after consideration of risk and resource availability. Various pathogen reduction methods have also been proposed to reduce the risk of TT-HEV. Future studies are needed to define the incidence of transmission through transfusion, their clinical features, outcomes and prognosis.

Key Words: Hepatitis E virus; Acute and chronic hepatitis; Immunosuppression; Blood transfusion; Transplantation

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Grade C (Good): C
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Core Tip: Transfusion-transmitted hepatitis E virus (HEV) is an emerging global health concern. In immunocompromised patients, chronic HEV infection increases the risk of liver cirrhosis. The prevalence of viremia and anti-HEV immunoglobulin G in asymptomatic blood donors varies widely between countries but even low concentrations of HEV in blood components are infectious, and in most countries blood donations are not routinely screened for HEV. Treatment of persistent infection includes modification of the immunosuppressant regimen followed by ribavirin. The need for screening of HEV in all blood donations remains controversial. Strategies to reduce de novo HEV infection should also be emphasized.

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INTRODUCTION

Hepatitis E virus (HEV) was first discovered as an epidemic of non-A, non-B hepatitis in the 1980s[1], and has since become one of the major global causes of viral hepatitis. The World Health Organization estimated that HEV caused approximately 44000 deaths in 2015, and accounted for 3.3% of global deaths related to viral hepatitis[2]. A recent meta-analysis concluded that approximately 939 million of the global population have ever experienced HEV infection, and 15 to 110 million individuals have recent or ongoing infection[3]. The infection is generally self-limiting; however, it poses a threat to some vulnerable patients resulting in a significant burden of inpatient admissions, chronic infection, organ failure, and death[4]. The mortality rate can be greater than 20% in patients with chronic liver disease, cirrhosis, or pregnancy [4,5]. With a high HEV serological prevalence among the global population, the safety of blood products has become a public health concern. Herein, we review existing evidence on transfusion-transmitted HEV (TT-HEV), and the implications for screening of blood donations.

VIROLOGY

HEV is a positive-sense, single-stranded RNA icosahedral virus belonging to the genus *Orthohepevirus* within the Hepeviridae family[6]. *Orthohepevirus A* has eight distinct genotypes, of which HEV-1, -2, -3 and -4 infect humans[7]. HEV genotype C1, belonging to the species *Orthohepevirus C*, circulates in rats and can cause cross-species infection and sporadic zoonotic transmission to humans[8].

HEV exists in urine or feces as non-enveloped virions encased by a capsid. It circulates in blood in a membrane-associated, quasi-enveloped form (eHEV) which is considered to be less contagious[9]. The entry mechanisms for HEV are not well characterized, but once the genomic RNA is uncoated and delivered to the cytosol, the replication cycle is initiated[10]. The viral release that initiates subsequent infection requires multivesicular bodies through endosomal sorting complexes required for transport[11].

EPIDEMIOLOGY

The prevalence rates of HEV antibody are higher in developing countries than in developed countries[12]. The highest anti-HEV immunoglobulin G (IgG) seropositivity rate has been reported in Africa with a mean of 21.76%, followed by Asia (15.80%), Europe (9.31%), North America (8.05%), South America (7.28%), and Oceania (5.99%). In addition, the reported anti-HEV immunoglobulin M (IgM) seroprevalence rate was 3.09%, 1.86%, 0.79%, 0.22% and 2.43% in Africa, Asia, Europe, North America, and South America, respectively[3].

Among the four major genotypes that can infect humans, HEV-1 and -2 are mostly found in developing countries including Asia, Africa, Latin America, and Mexico. Infection is mainly transmitted *via* fecally contaminated water, but occasionally also by person-to-person and vertical transmission[13]. Hepatitis E occurs as outbreaks as well as sporadic cases of acute hepatitis, with the preponderance of cases among adolescents and young adults. When stratified by age, the estimated incidence of HEV-1 and -2 infection is roughly between 0.5% and 1.0% for ages 0 to 15 years, with rates increasing to between 1.0% and 1.4% for ages 15 years to 20 years, then falling rapidly to a lower rate of 0.2% and below in individuals older than 30 years[14].

HEV-3 accounts for most of the autochthonous infection in developed countries while HEV-4 is mainly found in Asia and sporadically in Europe[15,16]. The reported seroprevalence of HEV-3 ranged from 0.6% to 52.5% in Europe, 6% in United States, 3 to 16% in United Kingdom and up to 52% in some regions of France[17]. HEV-3 and HEV-4 are zoonotic viruses which are frequently transmitted *via* food, close contact with animals, or transfusion of viremic blood units[18].

CLINICAL FEATURES AND EXTRAHEPATIC MANIFESTATIONS

The incubation period following exposure to HEV ranges from 2 to 6 wks. HEV infection commonly takes a clinically silent, asymptomatic course with around 5% to 30% of infected individuals developing acute hepatitis[19]. Symptoms of acute hepatitis include fever, malaise, anorexia, vomiting, followed by jaundice, tea-colored urine, and hepatomegaly[20]. It is then followed by a convalescent phase with gradual recovery within a few weeks in immunocompetent patients[21]. Acute liver failure is rare and occurs more frequently in middle-aged/elderly patients[22]. Fulminant hepatitis with fatal outcome is uncommon, but has been observed in pregnant women or in patients with pre-existing liver disease. The development of fulminant hepatitis appears to be related to host-specific factors rather than virus genotype, variants, or specific substitutions[23]. HEV superinfection may trigger liver decompensation in patients with chronic liver disease or cirrhosis, resulting in acute-on-chronic liver failure, which is associated with significant short-term mortality[24,25]. Further research is needed to clarify the clinical features, course of illness, and prognosis of patients with decompensated cirrhosis who develop HEV infection.

HEV-3 and HEV-4 can persist in immunocompromised patients resulting in chronic infection, defined as viral replication lasting for more than 3 to 6 mo[26]. It has been well described in patients after solid organ or stem cell transplant, hematology patients receiving chemotherapy, or HIV-infected patients[27-32]. The prevalence of anti-HEV IgG was about 11.6% and viral RNA was 2% in solid organ transplant recipients[33]. In solid organ transplant recipients who were positive for HEV RNA, more than 60% developed chronic hepatitis[33].

The natural history of chronic hepatitis E infection is not well understood[34]. In liver transplant recipients infected by HEV, histological analyses of liver biopsy revealed atypical morphology that is distinct from those in immunocompetent patients during early phases of infection[35]. Proliferation of, and cytokine production by, CD4+ and CD8+ T-cells were impaired in patients with persistent HEV viremia[36]. Chronic hepatitis E leads to liver fibrosis and cirrhosis. Cases of HEV-related hepatocellular carcinoma have been reported[37].

Although HEV predominantly infects hepatocytes, it may also affect other organs and present as extrahepatic manifestations. The mechanisms by which HEV can induce extrahepatic manifestations are not fully understood, but hypotheses include direct cytopathic tissue damage by extrahepatic replication, or immunological processes induced by an overwhelming host immune response[38]. Details of extrahepatic manifestations are shown in Table 1[39-44].

PREVALENCE IN BLOOD DONORS

Viremia

The prevalence of HEV RNA in blood donors varies around the world. (Table 2)[45-78]. Most countries have a low prevalence of HEV viremia, ranging from 0.0013% to 0.086%. A relatively higher rate of viremia was reported in Germany (0.12%) and China (0.281%)[49,70]. A meta-analysis of 10 studies from China showed a pooled prevalence of HEV RNA of 0.1%[79]. The actual prevalence might have been underes-

Table 1 Extrahepatic manifestations associated with hepatitis E virus infection

System	Extrahepatic manifestations
Neurological	Guillain-Barré syndrome (GBS)
	Neuralgic amyotrophy
	Neuropathy
	Bell's palsy
	Encephalitis
	Transverse myelitis
	Myositis
	Myasthenia gravis
	Pseudotumor cerebri
	Seizure
Renal	Decrease glomerular filtration rate
	Glomerulonephritis
	Nephrotic syndrome
	Mixed cryoglobulinemia
Hematological	Thrombocytopenia
	Hemolytic anemia
	Aplastic anemia
	Hemophagocytic syndrome
	Monoclonal gammopathy of uncertain significance (MGUS)
Others	Thyroiditis
	Pancreatitis
	Myocarditis
	Polyarthritis

timated as some studies included in the meta-analysis conducted RNA detection only in those donors who were positive for anti-HEV IgM or antigen[79].

The prevalence of HEV-3 and -4 is affected by dietary habits[80]. Consumption of raw pork tartare and undercooked pork liver may represent a relevant risk factor for HEV infection in Germany[49]. Regular consumption of pork meat and shellfish were also reported in the viremic donors in China[70].

Since 70% of infections with HEV-3 and -4 are asymptomatic[81], it can be difficult to identify infected blood donors, as viremia occurs primarily during the pre-icteric phase[82]. Katiyar *et al*[72] described anti-HEV IgG positivity in 60.5% of the tested donors in India and yet none of them were positive for HEV RNA. In India, human HEV is caused exclusively by the HEV-1 genotype, which causes brief hepatitis and seldom results in chronic infection[83,84]. The difference in endemicity between HEV genotypes may affect the propensity to cause symptomatic disease and viral persistence, which in turn influences the likelihood of viremia among blood donors.

Other factors influencing the reported prevalence of HEV viremia are the sensitivity and plasma pool size of the various nucleic acid test screening platforms used[85]. For example, 33 of 90 donations with a viral load of 20-750 IU/mL were positive when tested individually but missed in the pooled screening in a study by Hogema *et al*[57]. Delage *et al*[66] revealed a low prevalence (n = 11/50765) and viral loads of HEV-RNA in Canadian blood donors based on individual nucleic acid amplification techniques (NAT). They postulated that if pooled NAT was used, only two positive donations with viral loads > 1000 IU/mL would have been detected. The true frequency of viremia in blood donors in studies using pooled NAT could be underestimated due to a dilution effect. Vollmer *et al*[86] found that screening using individual NAT yielded an approximately 50% higher detection frequency compared with NAT of a mini-pool of 96 samples; nevertheless, samples exclusively positive for individual NAT had a

Table 2 Hepatitis E virus ribonucleic acid prevalence in donor, only studies include more than 1000 study subjects are included

Ref.	Country	Initial screening method	Number of donations screened	Number positive donations	Prevalence (95%CI)	HEV genotype: *n*/*N*	Median (range) viral load, IU/mL	Outcome of recipient
Europe								
Fischer *et al*[45], 2015	Austria	RT-PCR (plasma pool of 96 samples) with 95% LOD 11.6 IU/mL	58915	7	0.012%	3: 7/7	(2200 to 290000)	N/A
Vercouter *et al* [46], 2019	Belgium	RT-PCR (plasma pool of 6 samples) with 95% LOD 18.6 IU/mL	38137	7	0.018%	N/A	(153 to 8710)	N/A
Harritshøj *et al* [47], 2016	Denmark	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	25637	11	0.043% (0.02% - 0.07%)	3 (in 2 samples)	13 (unquantifiable to 920)	(1) Look-back testing was performed in 7 recipients; all were tested negative for HEV RNA and anti-HEV IgM; (2) No recipient developed transaminitis; and (3) One patient had strongly positive anti-HEV IgG assay which may indicate recent HEV infection or secondary immune response by HEV re-exposure.
Gallian *et al*[48], 2014	France	RT-PCR (plasma pool of 96 samples) with 95% LOD 23 IU/mL	53234	22	0.045% (0.043%-0.047%).	3c: 5/14; 3f: 8/14; 3 1/14	(468 to 5155800)	N/A
Westhölter *et al* [49], 2018	Germany	RT-PCR (plasma pool of 24 samples) with 95% LOD 18.6 IU/mL	18737	23	0.123%	3: 6/7	(120 to 11200000)	(1) Retrospective analysis of 4 viremic donors showed that they were HEV-positive in previous donations; (2) In 3 donors, testing of the previously donated blood in pools of 24 samples failed to identify viremic donations but were tpositive in unpooled samples; (3) Fourteen recipients had received HEV RNA positive blood products; (4) One immunosuppressed recipient tested positive for HEV RNA, developed acute on chronic liver failure, and died; and (5) One immunocompetent recipient developed acute self-limited episode of hepatitis E
Dreier *et al*[50], 2018	Germany	RT-PCR with 95% LOD 4.7 IU/ml for FFP, platelet concentrates, and RBC supernatant; 95% LOD 8.9 IU/mL for RBCs.	235524	182	0.077%	3: 4/4	(< 25 to 69.4)	(1) Nine viremic donations were transfused to 6 different recipients; (2) Two recipients were immunocompromised (heart transplantation and leukemia); (3) Two recipients died shortly after transfusion for reasons other than HEV infection; and (4) None of the other 4 recipients developed acute HEV infection or had detectable HEV RNA / anti-HEV IgG
Corman *et al*[51], 2013	Germany	RT-PCR (plasma pool of 96 samples mixed in metapools of 20)	93955	14	0.015%	3: 14/14	(3.1 to 4.8 Log_{10} IU/mL)	N/A
Vollmer *et al*[52], 2012	Germany	RT-PCR (plasma pool of 48 samples) with 95% LOD 4.7 IU/ml	16125	13	0.081%	3: 13/13	(13 to 68100)	N/A
Baylis *et al*[53], 2012	Germany	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	18,100	4	0.022%	3	(3.26 to 5.35 log_{10} copies/mL)	Donations screened positive for HEV were excluded from pharmaceutical production
O'Riordan *et al*	Ireland	TMA assay with 95% LOD	24985	5	0.020%	3: 3/3	(10 to 44550)	N/A

[54], 2016		5.5 IU/mL			(0.0065%-0.0467%)			
Spreafico *et al* [55], 2020	Italy	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	9726	1	0.010% (0.00%-0.06%)	N/A	N/A	N/A
Spada *et al*[56], 2018	Italy	RT-PCR, plasma pool and sensitivity varies according to anti-HEV IgG and IgM status	10011	0	N/A	N/A	N/A	N/A
Hogema *et al*[57], 2015	Netherlands	RT-PCR (plasma pool of 96 samples) with 95% LOD 38.4 IU/mL with the EasyMag extraction method and 10.3 IU/mL using Qiagen extracts	59474	41	0.069%	3c: 15/17; 3f: 2/17	N/A	N/A
Slot *et al*[58], 2013	Netherlands	RT-PCR (plasma pool of 48 or 480 samples) with 95% LOD 25 IU/mL	40176	13	0.032%	3: 16/17	(< 25 to 470000)	N/A
Grabarczyk *et al* [59], 2018	Poland	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	12664	10	0.079% (0.043%-0.145%)	3i: 2/3; 3c: 1/3	(16 to 6586 in 4 patients with positive results in qPCR)	N/A
Rivero-Juarez *et al* [60], 2019	Spain	RT-PCR (plasma pool of 8 samples) with sensitivity 670 IU/mL	11313	4	0.035% (0.01%-0.09%)	3: 4/4	(10788 to 2000000)	(1) Five patients received transfusions from HEV-infected donors; and (2) None of them showed an increase in alanine aminotransferase levels after transfusion
Sauleda *et al*[61], 2015	Spain	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	9998	3	0.030% (0.01%-0.09%)	3f (in 1 sample)	(250 to 2755)	N/A
Baylis *et al*[53], 2012	Sweden	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	95835	12	0.013%	3	(3.20 to 5.68 $\log_{10}$ copies/mL)	Donations screened positive for HEV were excluded from pharmaceutical production
Harvala *et al*[62], 2019	United Kingdom	RT-PCR (plasma pool of 24 samples) with 95% LOD 18.6 IU/mL	1838747	480	0.026%	3c: 112/149; 3e: 21/1493f: 12/149; 3a: 1/149; 2 distantly related to 3h, and 1 clustered distantly with 3a	883 (1 to 3230000)	N/A
Thom *et al*[63], 2018	United Kingdom	RT-PCR (plasma pool of 24 samples)	94302	38	0.040%	3: 10/10	N/A	N/A
Hewitt *et al*[64], 2014	United Kingdom	RT-PCR (plasma pool of 24 samples)	225000	79	0.035%	3: 79/79	3900 (50 to 2.37 × 10^6)	(1) Forty-three patients who had received blood components from HEV-infected donors were followed up; (2) The overall transmission rate was 42% (18 of 43 exposed patients); (3) One recipient developed clinical hepatitis and 4 recipients developed asymptomatic transaminitis; and (4) Four heavily

								immunosuppressed patients had delayed (37-38 wk) seroconversion or no antibodies detected
Cleland *et al*[65], 2013	United Kingdom	Nested PCR (plasma pool of 24 samples) with 95% LOD 201 IU/mL	43560	3	0.0069%	3: 3/3	N/A	N/A
North America								
Delage *et al*[66], 2019	United States	RT-PCR on individual samples with 95% LOD 18.6 IU/mL	50724	3	0.0059%	3: 2/3; genotyping was unsuccessful in 1 patient	(23 to 1420)	N/A
	Canada		50765	11	0.022%	3 (in 1 sample)	(< 10 to 3080)	
Roth *et al*[67], 2017	United States	RT-PCR (plasma pool of 96 samples) with 95% LOD 18.6 IU/mL	128021	4	0.003%	3a: 3/3	(3.0 to 3.8 log IU/mL)	N/A
Stramer *et al*[68], 2016	United States	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	18829	2	0.011% (0.0018%-0.351%)	N/A	14 IU/mL in one sample	N/A
Xu *et al*[69], 2013	United States	RT-PCR (plasma pool of 7 to 8 samples) with 95% LOD 400 IU/mL and nested PCR with 95% LOD 200 IU/mL	1939	0	N/A	N/A	N/A	N/A
Baylis *et al*[53], 2012	United States	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	51075	0	N/A	N/A	N/A	N/A
Asia								
Wen *et al*[70], 2018	China	RT-PCR on individual plasma	5345	15	0.281%	One 4h, another one clustered between genotype 2 and 4i	N/A	N/A
Tsoi *et al*[71], 2019	Hong Kong	RT-PCR with 95% LOD 7.89 IU/mL	10000	2	0.02%	4 (in 1 sample)	N/A	N/A
Katiyar H *et al* [72], 2018	India	RT-PCR (plasma pool of 3 samples) with LOD 100 IU/mL	1799	0	N/A	N/A	N/A	N/A
Minagi T *et al* [73], 2016	Japan	RT-PCR (plasma pool of 50 or 500 samples) with 95% LOD 152 IU/mL	620140	36	0.0058%	3: 36/36	(< 1.69 to 7.22 $\log_{10}$ copies/mL)	N/A
Intharasongkroh *et al*[74], 2019	Thailand	RT-PCR (plasma pool of 6 samples) with 95% LOD 53.5 IU/mL	30115	26	0.086%	3: 6/6	N/A	N/A

Others								
Hoad *et al*[75], 2017	Australia	TMA (plasma pool of 6 samples)	74131	1	0.0013%	N/A	180	N/A
Shrestha *et al*[76], 2016	Australia	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	14799	1	0.0068% (0.0002%-0.0376%	3	15000	N/A
Hewitt *et al*[77], 2018	New Zealand	RT-PCR (plasma pool of 8 to 12 samples)	5000	0	N/A	N/A	N/A	N/A
Maponga *et al* [78], 2020	South Africa	TMA assay on individual plasma with 95% LOD 7.9IU/mL	10000	1	0.01%	3	79000	All donations from donors with active HEV infection were discarded

CI: Confidence interval; FFP: Fresh frozen plasma; HEV: Hepatitis E virus; Ig: Immunoglobulin; LOD: Limit of detection; RBC: Red blood cells; RNA: Ribonucleic acid; RT-PCR: Real time polymerase chain reaction; TMA: Transcription mediated amplification.

corresponding viral load of < 25 IU/mL. High-sensitivity individual NAT can yield false-positive results[55]. Whether the identification of low-level HEV-positive donors translates into clinical significance and whether a single individual NAT is adequate remain undefined.

Antibodies

In addition to direct detection of HEV RNA, another important indirect assessment of HEV burden is the prevalence of anti-HEV IgM and IgG in blood donors (Table 3)[45,46,54-56,58,59,61,63,65,68,69,71,72,77,87-124]. HEV IgG prevalence increases with age which likely represents the cumulative effect of HEV exposure over a lifetime, especially as IgG antibodies can persist for decades[81]. The absence of detectable antibodies in donors was related to an increased risk of transfusion transmission of HEV[64]. However, the presence of anti-HEV IgG may not always be protective as multiple HEV reinfections could occur despite pre-existing antibodies[125]. Various HEV strains in serum are capable of replication in cell culture and generate infectious particles in the culture supernatant despite the coexistence of antibodies[126]. Anti-HEV IgM could be used to detect recent infection yet it failed to identify infected donors during the window period. For example, a meta-analysis of data from 28 countries found that only 26.6% of viremic blood units had positive anti-HEV antibodies[127]. In another study by Tedder *et*[128] *al*, a significant portion of viremic individuals (n = 57/79) were seronegative at the time of donation. Anti-HEV IgM sometimes exhibits unexpectedly long persistence for up to 3 years after a self-limiting acute hepatitis E episode[129]. Only a minority of anti-HEV IgM-positive donors have detectable RNA[58,93,103,109]. All these findings suggest that detection of anti-HEV IgG or IgM alone may not provide effective screening of HEV in blood donors.

Table 3 Seroprevalence of hepatitis E in blood donors

Ref.	Country	Number of donations screened	Assay used	Number of samples positive for HEV IgG antibodies	Anti-HEV IgG prevalence (95%CI)	Number of samples positive for HEV IgM antibodies	Anti-HEV IgM prevalence (95%CI)	Number of samples positive for HEV RNA in anti-HEV IgM positive	Viral load, IU/mL	Genotype
Europe										
Fischer *et al*[45], 2015	Austria	1203 (from HEV RNA negative donors)	Wantai	163	13.55% (11.6%-15.5%)	N/A	N/A	0	N/A	N/A
Vercouter *et al* [46], 2019	Belgium	356 (from HEV RNA negative donors)	Wantai	31	8.71% (6.20%-12.10%)	0	N/A	0	N/A	N/A
Miletić *et al*[87], 2019	Croatia	1036	3 commercial ELISA assays were used, only findings with highest prevalence are shown	209	20.17%	46	4.44%	0	N/A	N/A
Holm *et al*[88], 2015	Denmark	504	In-house NIH assay	54	10.7% (8.2%-13.7%)	N/A	N/A	N/A	N/A	N/A
			Wantai	100	19.8% (16.4%-23.6%)					
Dimeglio *et al* [89], 2018	France	300	Wantai	23	7.7% (4.9%-11.3%)	2	0.6% (0.1%-2.4%)	0	N/A	N/A
Juhl *et al*[90], 2013	Germany	1019	RecomWell assay and Western blot	69	6.8% (5.3%-8.3%)	N/A	N/A	N/A	N/A	N/A
Dalekos *et al* [91], 1998	Greece	3016	Abbott assay and Western blot	8	0.27%	0	N/A	N/A	N/A	N/A
O'Riordan *et al* [54], 2016	Ireland	1076	Wantai	57	5.3% (4.0%-6.8%)	2	0.19%	0	N/A	N/A
Spreafico *et al* [55], 2020	Italy	767	DiaPro	52	6.8% (5.1%-8.8%)	0	N/A	0	N/A	N/A
Spada *et al*[56], 2018	Italy	10011	Wantai	869	8.7% (8.14%-9.25%)	46	0.4% (0.34% - 0.61%)	0	N/A	N/A
De Sabato *et al* [92], 2017	Italy	170	Bio-Chain Institute and Western blot	15	8.82%	3	1.76%	0	N/A	N/A
Lucarelli *et al* [93], 2016	Italy	313	Wantai	153	48.9% (43%-54%)	2	0.6% (0.08%-2.3%)	1	100	3
Puttini *et al*[94], 2015	Italy	132	EIAgen HEV IgG kit	12	9.1%	N/A	N/A	N/A	N/A	N/A

Hogema *et al* [95], 2014	Netherlands	513	Wantai	58	11.31%	N/A	N/A	N/A	N/A	N/A
Slot *et al*[58], 2013	Netherlands	5239	Wantai	1401	26.7% (25.6%-28.0%)	49	0.94%	4	Range: < 25 to 3700	3
Grabarczyk *et al* [59], 2018	Poland	3079	Wantai	1340	43.52% (41.78%-45.28%)	39	1.27% (0.93%-1.73%)	N/A	N/A	N/A
Sauleda *et al* [61], 2015	Spain	1082	Wantai	216	19.96% (17.60%-22.32%)	13	1.20%	0	N/A	N/A
			Mikrogen	116	10.72% (8.90%-12.60%)					
Mateos *et al*[96], 1999	Spain	863	Abbott assay and Western blot	34	3.9%	0	N/A	N/A	N/A	N/A
Niederhauser *et al*[97], 2018	Switzerland	3609	Wantai	737	20.4% (19.1%-21.8%)	N/A	N/A	N/A	N/A	N/A
Kaufmann *et al* [98], 2011	Switzerland	550	MP Biomedicals	27	4.9%	N/A	N/A	N/A	N/A	N/A
Thom *et al*[63], 2018	United Kingdom	1714	Wantai	104	6.1% (5.0%-7.3%)	N/A	N/A	N/A	N/A	N/A
Cleland *et al*[65], 2013	United Kingdom	1559	Wantai	73	4.7% (3.6%-5.8%)	0	N/A	N/A	N/A	N/A
Beale *et al*[99], 2011	United Kingdom	262	Wantai	31	11.8%	4	1.5%	0	N/A	N/A
North America										
Zafrullah *et al* [100], 2018	United States	5040 (from HEV RNA negative donor)	DSI	569	11.29%	146	2.90%	0	N/A	N/A
			MP Biomedicals	537	10.65%	93	1.85%			
			Wantai	619	12.28%	34	0.67%			
Stramer *et al* [68], 2016	United States	4499	MP Biomedicals	329	7.3% (6.6%-8.1%)	26	0.58% (0.39%-0.85%)	N/A	N/A	N/A
Xu *et al*[69], 2013	United States	1939	Wantai	364	18.8% (17.0%-20.5%)	8	0.4% (0.1%-0.7%)	0	N/A	N/A
South America										
Di Lello *et al* [101], 2020	Argentina	391	DiaPro	44	11.3%	8	2.0%	0	N/A	N/A
Bangueses *et al* [102], 2020	Uruguay	400	DiaPro	40	10%	19	4.75%	3	N/A	3

Asia										
Nouhin *et al* [103], 2016	Cambodia	301	Wantai	85	28.2% (23.4%-33.5%)	3	1.0% (0.01%-1.8%)	1	956	3
Chen *et al*[104], 2019	China	4044	Wantai	799	19.8% (18.6%-21.0%)	43	1.1% (0.8%-1.4%)	2	N/A	4
Wen *et al*[70], 2018	China	5345	Wantai	1227	22.96%	38	0.71%	15	N/A	N/A
Wang *et al*[105], 2017	China	9069	Wantai	2682	29.57%	131	1.44%	5	N/A	N/A
Ma *et al*[106], 2015	China	816	Wantai	172	21.1%	4	0.5%	0	N/A	N/A
Ren *et al*[107], 2014	China	10741	Wantai	2945	27.42%	109	1.01%	0	N/A	N/A
Zhuang *et al* [108], 2014	China	486	ELISA based on antigen protein pB166 and MPII	113	23.3%	N/A	N/A	N/A	N/A	N/A
Tsoi *et al*[71], 2019	Hong Kong	2000	Wantai	315	15.8% (14.2%-17.4%)	16	0.8%	0	N/A	N/A
Tripathy *et al* [109], 2019	India	2447	Wantai	433	17.70% (16.23%-19.26%)	5	0.20%	2	Ranged from 3.5×10^4 to 4.6×10^5 copies/mL	1
Katiyar *et al*[72], 2018	India	633	Wantai	383	60.5%	N/A	N/A	0	N/A	N/A
Gajjar *et al*[110], 2014	India	460	DiaPro	N/A	N/A	22	4.78%	N/A	N/A	N/A
Parsa *et al*[111], 2016	Iran	700	DiaPro	42	6.0%	5	0.71%	5 (only 50 seropositive blood donors were tested)	N/A	1
Hesamizadeh *et al*[112], 2016	Iran	559	DiaPro	45	8.05%	N/A	N/A	N/A	N/A	N/A
Naeimi *et al* [113], 2015	Iran	628	HEV IgG, Pasto, Iran	105	16.72%	N/A	N/A	N/A	N/A	N/A
Ehteram *et al* [114], 2013	Iran	530	DiaPro	76	14.3%	N/A	N/A	N/A	N/A	N/A
Taremi *et al* [115], 2007	Iran	399	DiaPro	31	7.8%	N/A	N/A	N/A	N/A	N/A
Takeda *et al* [116], 2010	Japan	12600	in-house ELISA	431	3.42%	N/A	N/A	N/A	N/A	N/A
Shrestha *et al*	Nepal	1845	Wantai	773	41.9% (39.7%-	55	3.0% (2.2%-3.8%)	N/A	N/A	N/A

[117], 2016					44.2%)					
Nasrallah *et al* [118], 2017	Qatar	5854	Wantai	1198	20.46%	34	0.58%	4	N/A	N/A
Jupattanasin *et al* [119], 2019	Thailand	630	EUROIMMUN test kit	187	29.7% (26.2%-33.4%)	N/A	N/A	N/A	N/A	N/A
Africa										
Traoré *et al*[120], 2016	Burkina Faso	1497	DiaPro and Wantai	584	39%	13	0.87%	N/A	N/A	N/A
Ibrahim *et al* [121], 2011	Egypt	760	N/A	N/A	N/A	3	0.39%	2	N/A	N/A
Meldal *et al* [122], 2013	Ghana	239	4 commercial assays were used, findings reactive in; at least two serological assays are shown	11	4.6%	14	5.9%	0	N/A	N/A
Lopes *et al*[123], 2017	South Africa	300	Fortress Diagnostics	76	25.3%	0	N/A	N/A	N/A	N/A
Ben-Ayed *et al* [124], 2015	Tunisia	426	Globe; Diagnostics Srl ELISA	19	4.46%	N/A	N/A	N/A	N/A	N/A
Others										
Hewitt *et al*[77], 2018	New Zealand	1013	Wantai	98	9.7% (7.9%-11.7%)	N/A	N/A	N/A	N/A	N/A
			MP Biomedicals	82	8.1% (6.5%-10.0%)					

ALT: Alanine aminotransferase; CI: Confidence interval; DSI: Diagnostic Systems Incorporated; ELISA: Enzyme-linked immunosorbent assay; HEV: Hepatitis E virus; NIH: National Institutes of Health.

Geographical variation, racial differences, and diverse study methodology and laboratory techniques all contribute to differences in HEV seroprevalence. More than one-third of donors had evidence of past HEV infection in Poland, India, Nepal and Burkina Faso[59,72,117,120]. Lucarelli *et al*[93] reported an unexpectedly high prevalence (48.9%) of anti-HEV IgG among 313 donors in central Italy. Eating raw dried pig liver sausage was the only independent risk factor for HEV IgG in their study, but the authors speculated that the uncontrolled expansion of the wild boar population had resulted in contamination of the soil and watercourses for people living in rural areas, and this may also have also contributed to the high prevalence of HEV[93].

Caution is needed when interpreting the HEV serology results because commercial kits for serological detection show marked variation in sensitivity and specificity. Despite the relatively high sensitivity of the IgM assay, the sensitivity of IgG detection kits is highly dependent on a patient's immune status, being 80% to 90% in immunocompetent individuals, but falling dramatically to 15% to 45% in immunocom-

promised patients[130]. In a meta-analysis conducted in Europe, the pooled anti-HEV IgG seroprevalence rates determined by different commercial assays showed large variability with reported seroprevalence rates ranging from 2% to 17%[131]. Poor concordance of test results between the Wantai, Dia.Pro and MP Diagnostics HEV enzyme-linked immunosorbent assays (ELISA) were observed[132,133]. This may partly explain the broad ranges of anti-HEV IgG prevalence (5.3% to 48.9%) reported in Italy[55,56,92-94]. In contrast, most studies conducted in China used the Wantai assay and revealed a similar seroprevalence of around 20% to 30%. This assay is believed to be more sensitive than other commercial assays in detecting anti-HEV IgG [134,135].

TRANSFUSION-TRANSMITTED HEPATITIS E

HEV transmission *via* transfusion has been reported since 2004[136] and there has been increasing recognition of the risk of transmitting HEV by transfusion in recent years. Cases of TT-HEV are shown in Table 4[137-150]. Identical genomic sequences were identified in most infected patients and blood donors. Table 4 Likely only represents the tip of the iceberg as other probable or possible cases have been reported in the literature[151,152]. At the same time, patients with mild symptoms of hepatitis E may have gone undiagnosed. Physicians should stay vigilant for HEV infection in patients who have received a blood transfusion.

Although blood components that contain larger plasma volumes, principally fresh frozen plasma and platelet components, are believed to transmit HEV more readily [64], a number of TT-HEV cases associated with red blood cell transfusion have also been described[138,140,141,143,144,148-150]. Red blood cell transfusion was a significant risk factor for HEV seropositivity in patients on hemodialysis in Croatia[153]. Twenty percent (n = 8/40) of multiply transfused thalassemia patients were anti-HEV IgG positive compared with 11.0% (n = 10/91) in blood donors[154]. In contrast, a study in Iran found anti-HEV antibodies in only 1.67% of patients with thalassemia, suggesting a low rate of TT-HEV in that country[155]. Results from these two studies in thalassemia patients were limited by the small sample size. Ankcorn *et al*[156] analyzed 1591 patients with hematologic malignancy and found that the more transfusions of non-HEV screened blood products the patients had received, the higher their likelihood of being IgG seroreactive was, suggesting HEV acquisition *via* transfusion in these patients.

A study by Hewitt *et al*[64] indicated that a viral concentration of between 407 and 257039 IU/mL in blood products was associated with TT-HEV, and that a high viral load in donors rendered infection more likely (P < 0.0001). However, this may not be true in immunocompromised patients. In a systematic review, Dreier *et al*[50] calculated the median transfused viral load in HEV-infected and non-infected immunocompromised patients. Although the transfused viral load was higher in the infected than the non-infected individuals (4.80×10^5 IU *vs* 1.55×10^4 IU), the between-group difference was not statistically significant (P = 0.1006)[50]. A potential reason for this finding is that a low viral concentration (150 IU/mL) of the blood component could already be infectious[140].

Most cases of TT-HEV occur in immunocompromised recipients, such as patients with hematologic malignancies, or recipients of solid organ or hematopoietic stem cell transplants. However, patients on simple immunosuppressants like corticosteroids and cyclosporine or even immunocompetent individuals are also at risk[157]. Massive transfusion increased the risk of HEV transmission in an immunocompetent trauma patient[158]. Spontaneous resolution, viral eradication by immunosuppressant reduction and/or ribavirin are possible[159] but occasionally there are cases which have progressed into chronic hepatitis, liver cirrhosis or multi-organ failure. Transfusion recipients are more vulnerable to chronic liver injury than the general population as a result of foodborne infection[140]. More than 60% (n = 56/85) of solid organ transplant recipients infected with HEV developed chronic hepatitis, with tacrolimus use as an independent predictive factor[160]. Pas *et al*[161] screened 1200 solid-organ transplant recipients in the Netherlands for HEV RNA and identified 12 patients with HEV infection. Nine of these 12 patients had been treated with a tacrolimus-based regimen postoperatively. In liver transplant recipients, graft hepatitis with rapid histological disease progression and requirement of re-transplantation due to liver cirrhosis has been reported[162,163]. The rapid progression of HEV infection to advanced fibrosis and cirrhosis has also been observed in individuals receiving kidney or heart transplants[33]. In 50 patients with hematologic malignancy and clinically

Table 4 Reported cases of transfusion transmitted hepatitis E

Study	Number of patients	Comorbidity	Blood component received (*n*)	Viral load of transfused blood product IU/mL	Genotype	Treatment	Outcome
Okano *et al* [137], 2020	1	AML on chemotherapy	Plt	N/A	3b	Nil	Spontaneous resolution and developed HEV antibodies after cessation of chemotherapy for AML
Gallian *et al* [138], 2019	23	Solid organ transplant, *n* = 9; allogeneic hematopoietic stem cells transplant, *n* = 4; hematologic malignancies, *n* = 5; immunosuppressant, *n* = 2; immunocompetent, *n* = 3	RBC *n* = 7; apheresis Plt *n* = 3; whole blood-derived pooled Plt *n* = 6; FFP *n* = 7	Ranged from 1.14 × 10^3 to 31 × 62.10 ^{6}	3a, *n* = 1; 3c, *n* = 4; 3f, *n* = 16; 3, *n* = 1; 4d, *n* = 1	Ribavirin, *n* = 15	Acute HEV infection, *n* = 8; spontaneous resolution, *n* = 4; ribavirin treatment, *n* = 3; immunosuppressant reduction, *n* = 1; chronic HEV infection, *n* = 14, all immunosuppressed; resolution with ribavirin, *n* = 10; resolution with immunosuppressant reduction, *n* = 4; One solid organ transplant recipient did not clear HEV infection despite ribavirin and died of multiorgan failure
Ledesma *et al* [139], 2019	2	Allogeneic BMT, *n* = 1; liver transplant, *n* = 1	Plt	3 × 10^4	3e	Ribavirin, *n* = 1	The patient received BMT remained HEV-infected and IgM/IgG-negative until death; the patient with liver transplant was treated successfully with a course of ribavirin
Satake *et al* [140][a], 2017	19	Hematologic malignancies, *n* = 6; organ transplant, *n* = 2; systemic disease, *n* = 8; no major comorbidity, *n* = 3	RBC *n* = 10; Plt *n* = 6; FFP *n* = 3	Ranged from 1.5 × 10^2 to 5.3 × 10^6	4, *n* = 2	N/A	Two patients with malignant lymphoma and two who had received liver transplant developed chronic hepatitis E; the two liver transplant recipients were successfully cleared of HEV by ribavirin
Lhomme *et al* [141], 2017	3	Solid organ transplant	One patient received RBC; one patient received RBC and Plt; one patient received Plt and FFP	Ranged from 3.6 to 8.2 log IU	3, *n* = 1; 3f, *n* = 2	N/A	N/A
Yamazaki *et al*[142], 2017	2	Hematologic malignancies treated with chemotherapy	N/A	N/A	3b	N/A	Did not become chronic hepatitis E
Belliere *et al* [143], 2017	1	Heart transplant	RBC	1430 copies/mL	3	Ribavirin	Died from multi-organ failure despite treatment
Riveiro-Barciela *et al* [144], 2017	1	Immunocompetent, admitted for disseminated infection	RBC	75000	3	Nil	Spontaneous resolution
Hoad *et al* [145], 2017	1	Liver transplant	FFP	947	3	Ribavirin	Resolved with treatment
Matsui *et al* [146], 2015	1	AMI post CABG with hemorrhagic cardiac tamponade	Plt	$10^{6.8}$ copies	3	Nil	Spontaneous resolution
Huzly *et al* [147], 2013	1	Immunocompromised	Apheresis Plt	30888-37273	3f	N/A	N/A
Coilly *et al* [148], 2013	1	Liver transplant	RBC	3.5 $\log_{10}$	3c	Ribavirin	Resolved with treatment
Boxall *et al* [149], 2006	1	Lymphoma on chemotherapy	RBC	N/A	3	Nil	Spontaneous resolution

Mitsui *et al* [150], 2004	1	Hemodialysis	RBC	N/A	3	Nil	Subclinical infection without elevated ALT

[a]Two cases were not confirmed by sequence identity and should only be considered as probable TT-HEV.

ALT: Alanine aminotransferase; AMI: Acute myocardial infarction; AML: Acute myeloid leukemia; BMT: Bone marrow transplant; CABG: Coronary artery bypass graft; FFP: Fresh-frozen plasma; HEV: Hepatitis E virus; Ig: Immunoglobulin; Plt: Platelet concentrates; RBC: Red blood cell.

overt hepatitis E, the mortality rate was 16% (n = 8), with liver-related death occurring in 4 patients[164]. HEV could actively suppress the cellular immune response and increase levels of immunosuppressive interleukin-10 that may perpetuate chronic infection and subsequent liver damage[165,166].

TREATMENT

The management strategy for HEV infection should be determined by the clinical presentation. Currently, there is limited information in the published literature that describes the clinical features of TT-HEV, or the optimal approach to management. Acute TT-HEV infections are usually subclinical or mild, with no severe or fulminant cases reported[140]. Therefore, most acute HEV infections should be treated conservatively, while waiting for spontaneous clearance, although a short course of ribavirin may also be considered. In 21 patients with acute HEV infection who were at high risk of liver failure, receiving immunosuppressive therapy for an autoimmune disease or undergoing chemotherapy, a short course of ribavirin for up to 3 mo was associated with rapid virological response and normalization of liver enzymes[167].

The current practice for management of chronic HEV infection is mainly based on observational data[18]; Figure 1 shows a proposed algorithm for management. In patients who are on immunosuppressants, the first-line intervention should be a dose reduction or discontinuation of the immunosuppressive drug[168,169]. In solid organ transplant recipients, reducing the dose of immunosuppressive therapies that principally target T-cells can achieve HEV clearance in nearly one third of patients [160]. Most immunosuppressive drugs such as cyclosporine and tacrolimus increase HEV replication in vitro; mycophenolate mofetil is the only immunosuppressant agent demonstrated to have an anti-viral effect[170].

If modification of the immunosuppressant regimen is not possible or is unsuccessful, pharmacological agents such as ribavirin and/or pegylated interferon-alpha (peg-IFN) can be used[171]. In a meta-analysis that included 395 patients with chronic hepatitis E, ribavirin monotherapy for a median of 3 mo achieved sustained virological response (SVR) in 76% of patients[172]. The reported dose of ribavirin in the literature ranged from 29 to 1200 mg/d, and the duration from 1 to 18 mo. Data on the optimal treatment regimen are needed[173]. HEV RNA should be assessed in the serum and in the stool before treatment discontinuation[169]. A second course of ribavirin for 6 mo

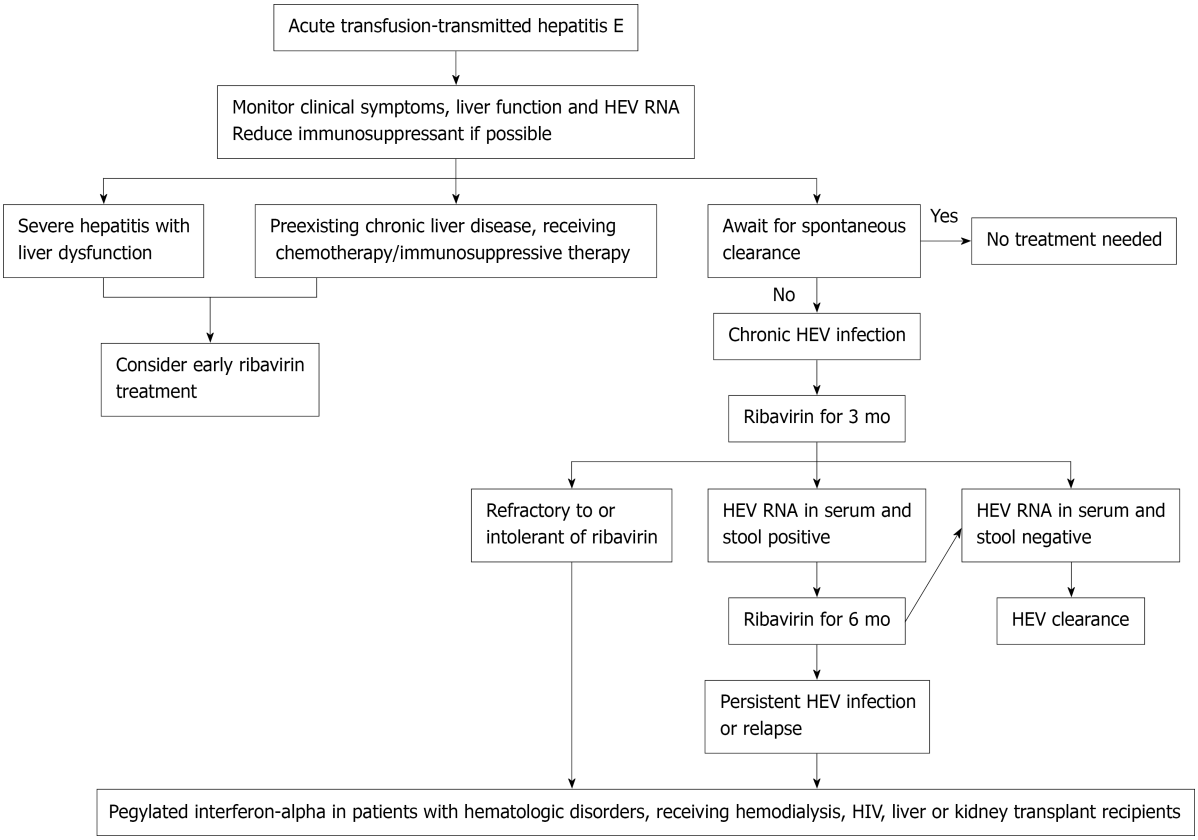


Figure 1 Recommended algorithm for management of transfusion-transmitted hepatitis E.

can be attempted in cases of treatment failure[172]. HEV RNA concentrations decrease within the first week of initiating ribavirin therapy, and a greater reduction in viral load on day 7 is an independent predictor of SVR[174]. Ribavirin failure has been linked to the presence of certain single nucleotide variants (SNVs) and in-frame insertions in the hypervariable region of open reading frame (ORF) 1 in the HEV genome[175].

For those who are refractory to, or intolerant of, ribavirin, peg-IFN can be considered. Its efficacy has been documented in patients with hematologic disorders, patients receiving hemodialysis, and in combination with ribavirin in patients with HIV[176-178]. Close monitoring is needed if it is used in transplant recipients because of an increased risk of acute humoral and cellular rejection[179,180]. Peg-IFN was thought to be safe only in liver transplant recipients until recent case reports described its successful use in a kidney transplant recipient[181-183].

Sofosbuvir is a nucleotide analog shown to decrease replication of HEV-3 *in vitro* [184]. However, in clinical studies, only modest antiviral activity was observed and SVR was not achieved[185-187]. Rescue treatment for patients who are not eligible for, or not responding to, ribavirin and/or peg-IFN remains an unmet need.

HOW TO REDUCE TRANSFUSION-TRANSMITTED HEPATITIS E

The background risk of foodborne HEV transmission to both donors and recipients of blood products is not negligible. The transfusion-related risk of infection only exceeds the annual dietary risk when more than 13 individual donor components are transfused[188]. Strategies to reduce *de novo* infection, such as modifying eating habits and eliminating HEV from pigs and other animals that are used for food production are essential[189]. The one available vaccine (HEV 239, Hecolin, Xiamen, China) is licensed only in China, and has yet to play a fundamental role in global outbreaks or pandemic control[190]. Nonetheless, the transmissibility and disease phenotype may not be the same for a person who acquires the virus orally and a person who gets infected intravenously, as there may be some protection provided by the acidic environment of the stomach and the mucosal barrier in the gut[191]. The infectivity of the non-enveloped form is different to that of enveloped HEV[9]. Data reporting

outcomes of recipients of HEV-infected blood products are sparse[47,49,50,60,64].

Policies on screening HEV in blood products differ between countries. Universal screening was adopted in the United Kingdom, Ireland, and the Netherlands. Germany and France implemented targeted screening of donated plasma intended for use in high-risk patients[192]. In Japan, the use of nucleic acid-based screening is limited to Hokkaido[193]. Blood donors are not routinely tested for HEV infection in China including Hong Kong[70,71,194]. There has been much debate on mandatory HEV screening in blood donations[195]. Key questions, such as whether or not to screen, which laboratory assay to use, which donors to screen (universal or selective screening), and which types of blood components to screen should be assessed based on risk assessment, resource availability, health economics, and political or other influences. The answers may vary considerably by geographical location[169,196]. In areas where HEV is highly endemic, most donors and/or recipients have probably been exposed to HEV previously and would have positive IgG antibodies. Therefore, the decision on serological screening should also take into consideration the prevalence of HEV infection in that particular region.

All donors should answer a questionnaire about symptoms of clinical hepatitis and potential exposure to HEV prior to blood donation. Donation should be deferred in any donors with a history of clinical hepatitis[197]. Neither alanine aminotransferase (ALT) nor anti-HEV IgM testing correlate with the presence of HEV RNA, supporting the use of NAT for screening of blood donations[60,61,105]. A simulation study by Kamp *et al*[198] reported that testing for HEV RNA by NAT with a pool size of 96, and a 95% limit of detection of 20 IU/mL will result in an 80% reduction in expected HEV transmissions as well as of consequent chronic infections with severe complications. The risk of transmission could be reduced by 90% in NAT using a mini-pool of 24 samples[198].

If opting for selective screening instead of universal screening, a clear definition of at-risk patients is warranted[199]. Targeted screening should be contemplated for blood components that will be supplied to transplant recipients, or patients with hematologic malignancies or chronic liver disease, as these individuals are at high risk of developing fulminant hepatitis, acute on chronic liver failure, or chronic hepatitis. However, it is not yet clear whether patients with rheumatologic diseases, those on low-intensity immunosuppression, or elderly individuals should only receive HEV-negative blood products. A multicenter retrospective study in Europe including 21 rheumatology and internal medicine patients found that patients with rheumatoid arthritis who were receiving methotrexate or biologics were at risk of chronic hepatitis E infection[200]. However, another study in France did not find worse hepatitis E severity or increased risk of chronicity in 23 patients with inflammatory arthritis treated with immunosuppressants[201].

Patients co-infected with HIV with CD4+ count < 200/mm^3 are at risk for persistent HEV infection[29]. In HIV patients with low CD4+ count, anti-HEV IgG seroconversion was delayed until immune reconstitution occurred[202]. A recent meta-analysis found that the HEV RNA positivity rate was significantly higher in transplant recipients than in HIV-positive patients [1.2% (95%CI: 0.9-1.6) *vs* 0.39% (95%CI: 0.2-0.7); $P = 0.0011$], possibly due to better immune status in the HIV-positive individuals using anti-retroviral therapy[203].

HEV-1 and -2 infections can take a fulminant course in pregnancy, resulting in liver failure, membrane rupture, spontaneous abortions, and stillbirths[204]. HEV-3 infection in pregnancy appears to be less virulent without significant maternal, fetal, or neonatal complications[205-207]. During pregnancy, a reduced cellular immunity and a high level of steroid hormones, in particular estrogen, progesterone, and human chorionic gonadotropin, influence viral replication/expression and possibly explain the disease severity[208]. The immune response could be influenced by HEV genotype, translating into different outcomes[209]. Ribavirin and peg-IFN are contraindicated in pregnancy due to concerns of teratogenicity[210]. Further studies are needed to clarify the risk of transmission of HEV to pregnant women *via* blood transfusion; however, in view of the potentially serious disease course and absence of a safe treatment, pregnant women are a priority group for HEV-negative blood products.

Roth *et al*[67] evaluated the safety of plasma-derived medicinal products (PDMP) and found a very low prevalence of HEV RNA (0.002%) in plasma donors. Since viral reduction methods are used in the manufacturing processes of PDMP, these data do not support routine screening of all plasma pools intended for producing PDMP. Currently there is a lack of evidence to suggest that human serum albumin or coagulation factor concentrates are a major source of HEV infection[211,212].

The cost effectiveness of HEV screening of blood donations was analyzed in the Netherlands. Screening of whole blood donations in pools of 24 would prevent 4.52 of the 4.94 TT-HEV infections annually at a cost of approximately €310000 (Euro) per prevented chronic case. The estimated cost per incurable case prevented was 10-fold higher. Costs could potentially be reduced by 85% if only the blood products intended for use by immunocompromised patients were screened. Additional costs for selective screening may arise for logistic reasons and a possible increase in the number of blood products that expire before use. They concluded that preventing HEV transmission by screening of blood donations appears not excessively expensive compared with other blood-screening measures but the impact on disease burden may be small as only a minority of all HEV cases are transmitted by blood transfusion[213]. Another economic analysis performed in North America found a very low estimated risk of TT-HEV infection risk leading to severe liver disease. When compared with no screening, the costs were $2.68 (USD) per component for a selective screening approach, and $6.68 per component for universal screening. The respective costs per quality-adjusted life-year gained were $225546 and $561810, respectively, which exceeded the threshold for what is considered as "cost-effective"[66].

In addition to screening, various pathogen reduction methods have been proposed to reduce risk of TT-HEV. Solvent/detergent treatment could not eliminate non-enveloped HEV in plasma[214]. Non-enveloped HEV is also resistant to the Intercept method, which combines a synthetic psoralen amotosalen HCl treatment with ultraviolet A light illumination to block the replication of DNA and RNA[215]. However, substantial viral reduction has been demonstrated during the manufacturing process of plasma products using immunoaffinity chromatography, nanofiltration, cold ethanol fractionation and heat treatment[216]. Anti-HEV antibodies enhanced HEV removal by nanofiltration[217]. Furthermore, ultraviolet C light provided effective inactivation of HEV in platelet concentrates[218].

CONCLUSION

To conclude, TT-HEV is gaining attention worldwide. Although the overall prevalence of viremic blood donations is low, HEV can cause sinister consequences in immunocompromised recipients. Future studies are needed to define the incidence of transmission through transfusion, clinical features, outcomes, and prognosis. The decision on a screening policy in asymptomatic blood donors should be based on local risk assessment and health economics.

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REVIEW

Viral hepatitis in 2021: The challenges remaining and how we should tackle them

Rebecca Dunn, Aaron Wetten, Stuart McPherson, Mhairi C Donnelly

ORCID number: Rebecca Dunn 0000-0003-0559-5755; Aaron Wetten 0000-0003-4989-9726; Stuart McPherson 0000-0002-5638-2453; Mhairi C Donnelly 0000-0001-7655-7284.

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Rebecca Dunn, Gastroenterology, University Hospital of North Tees, Stockton on Tees TS198PE, United Kingdom

Aaron Wetten, Stuart McPherson, Mhairi C Donnelly, Liver Unit, Freeman Hospital, Newcastle NE77DN, United Kingdom

Aaron Wetten, Stuart McPherson, Translational and Clinical Research Institute, Newcastle University, Newcastle NE17RU, United Kingdom

Corresponding author: Mhairi C Donnelly, MBChB, Doctor, Liver Unit, Freeman Hospital, Freeman Road High Heaton, Newcastle NE77DN, United Kingdom. mhairi.donnelly@nhs.net

Abstract

Viral hepatitis results in 1.4 million deaths annually. The World Health Organization (WHO) set an ambitious target to eliminate viral hepatitis by 2030, but significant challenges remain. These include inequalities in access to healthcare, reaching at risk populations and providing access to screening and effective treatment. Stigma around viral hepatitis persists and must be addressed. The WHO goal of global elimination by 2030 is a worthy aim, but remains ambitious and the coronavirus 2019 pandemic undoubtedly has set back progress. This review article will focus on hepatitis A to E, highlighting problems that have been resolved in the field over the past decade, those that remain to be resolved and suggest directions for future problem solving and research.

Key Words: Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis D; Hepatitis E; COVID-19

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Core Tip: Viral hepatitis results in 1.4 million deaths annually. The World Health Organization set an ambitious target to eliminate viral hepatitis by 2030, but significant challenges remain. These include inequalities in access to healthcare, reaching at risk populations and providing access to screening and effective treatment. In this review article, we discuss the advances in the field of viral hepatitis over the past decade. We also discuss the remaining challenges relating to viral hepatitis A to E, and suggest strategies and pathways for their resolution.

Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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INTRODUCTION

Our understanding of the epidemiology of viral hepatitis and associated treatment strategies has advanced significantly over the past decade. Arguably, the most significant advances have occurred in the treatment of chronic hepatitis C, which is now curable with a short course of all oral antiviral therapy. Despite this, viral hepatitis still kills more than 1.4 million people a year[1]. As such, viral hepatitis has become a global health priority and a number of large-scale public health policies have been implemented. The World Health Organization (WHO) has set out an ambitious global elimination strategy for viral hepatitis, aiming to eliminate viral hepatitis as a public health threat by 2030[2]. Key interventions for viral elimination have been identified and include hepatitis B vaccination, facilitation of safe injection practices and safe blood transfusions, promotion of safe sex, hepatitis B treatment and hepatitis C cure. However, modelling studies suggest that up to 80% of high-income countries will not meet the WHO target[3].

This review article will focus on hepatitis A-E, highlighting problems that have been resolved in the field over the past decade, those that remain to be resolved and suggest directions for future problem solving and research. We will also discuss the impact of the coronavirus 2019 (COVID-19) pandemic on viral elimination.

METHODS

A PubMed search was performed using the following terms: "hepatitis A"; "hepatitis B"; "hepatitis C"; "hepatitis D"; "delta agent"; "hepatitis E"; "cirrhosis"; "direct acting antivirals"; "chronic kidney disease"; "chronic liver disease"; "functional cure"; "hepatocellular carcinoma"; "liver transplant"; "reinfection"; "ribavirin"; "viral elimination"; "viral resistance"; "virologic cure". Only English-language articles were included in this review. Reference lists of selected articles were reviewed for relevant studies. Published abstracts were included.

HEPATITIS A VIRUS

Worldwide, the incidence of hepatitis A virus (HAV) is decreasing[4,5], but with increasing globalization there are significant shifts in the epidemiology of HAV infection[6]. Due to a large number of cases being asymptomatic and an estimated under-reporting of up to 80% of cases, it is acknowledged that the true incidence is difficult to quantify[7]. The incidence rate of HAV infection is strongly correlated with socioeconomic indicators; the incidence decreases with increasing access to clean water and sanitation. HAV infection is commonly reported in countries where conflict leads to the displacement of people, resulting in poor sanitation and overcrowding[8].

Advances in the past decade and problems now solved

Recent studies have expanded our understanding of the molecular virology and pathobiology of HAV. It is likely that multiple immune mechanisms contribute to the development of acute liver injury due to HAV infection, including decreased frequency of regulatory T-cells due to Fas-mediated apoptosis[9] and a polymorphism in TIM1[10]. Factors now recognized to influence the clinical course of HAV infection include variations in the viral nucleotide sequence within the 5'UTR[10].

The WHO estimated that HAV infection caused approximately 7134 deaths in 2016 [11]. In the United States, case-fatality estimates range from 0.3% to 0.6% for all age groups, rising to 1.8% amongst patients aged > 50 years[12]. A safe and effective inactivated vaccine has been in use for almost 30 years[13]. It was initially developed for individual prophylaxis, but now is used to control endemics[13]. A live attenuated

vaccine has been developed and licensed in China and it is used in the Chinese national vaccination program. Use of this vaccine in children has reportedly reduced the incidence of HAV infection by 80%[14]. There are now 34 countries that use or are planning to introduce HAV vaccination into routine immunization of children in specific risk groups[11]. Within the United Kingdom, persons who are considered high-risk for HAV infection and should be offered vaccination include those in close contact with someone with HAV infection, travelers who plan to travel to parts of the world where HAV is highly endemic, persons with chronic liver disease, men who have sex with other men (MSM), people who inject drugs (PWIDs) and those who are likely to be exposed to HAV from their employment, for example workers who are exposed to raw sewage such as within the construction industry.

Another advance in the past decade has been in the area of post-exposure prophylaxis (PEP) against HAV. PEP is recommended for persons who are immunocompromised and those who have chronic liver disease[15]. Immunoglobulin was previously the only recommended PEP however due to a number of factors including declining anti-HAV IgG titres in donor pools, new strategies were sought. Recent data support post-exposure immunization with an inactivated HAV vaccine as being effective in preventing infection when given within 14 d of exposure[13].

Problems remaining to be solved

Prevention of infection in high-risk populations (including targeted vaccination): With increasing numbers of forcibly displaced persons in certain parts of the world [16], endemic HAV infection will continue to be an ongoing but preventable issue that requires a global response to provide public health infrastructure, sanitation and free HAV vaccination programmes. This approach requires significant input from public health agencies and politicians alike.

Person to person transmission is described, with infection reported amongst PWIDs and homeless populations. These populations can be difficult to engage, and vaccinating these high-risk individuals needs to be a public health priority (at least in developed countries). MSM have been linked to outbreaks of cases in developed countries, with epidemiological and laboratory investigations linking genotypes between countries[17]. It is important that high-risk groups such as MSM are identified and offered vaccination to prevent outbreaks in susceptible communities where there is lack of herd immunity[18]. Improving uptake of HAV vaccination in the MSM population is a remaining challenge. Targeting these at risk populations by methods such as social media and dating apps have been shown to improve vaccination uptake[17]. Patients with chronic liver disease should also be offered HAV vaccination due to their risk of more severe infection, however doing so has not entered widespread clinical practice[19]. In one American study of HAV vaccination in patients with chronic liver disease, 28% of patients seen in specialist centres underwent vaccination compared with 5% of patients managed in primary care[20]. In another American study of patients with hepatitis C, 7.9% of patients underwent HAV vaccination[21]. As HAV is a vaccine-preventable disease, universal vaccination of infants would be an effective method for controlling the infection going forwards.

Treatment of severe liver injury due to HAV infection: Although rare, patients with acute HAV infection can progress to acute liver failure (ALF)[7]. Whilst these patients can recover with supportive management, a small number of patients may require transplantation. Patients progressing to ALF are typically older and may not be suitable candidates for liver transplantation, and therefore other specific treatment strategies are required. Furthermore, liver transplantation is not accessible to those most at risk in displaced communities. Ribavirin has successfully been used in treatment of acute hepatitis E infection; it has been shown to have an inhibitory effect on HAV *in vitro* but has not been assessed *in vivo* for therapeutic activity[22].

HEPATITIS B + D

Hepatitis B virus

Chronic hepatitis B infection is a global problem, but the burden of disease is mostly in low to middle income countries, with 248 million of the estimated 292 million people affected residing in Asia, Africa, the Pacific and Latin America. Chronic hepatitis B virus (HBV) accounts for approximately 47% of all viral hepatitis related deaths, the vast majority of which are secondary to complications of chronic liver disease[23,24].

Advances in the past decade and problems now solved

In 2017 the nomenclature to describe the different phases of chronic HBV changed within the updated European Association for the Study of the Liver (EASL) hepatitis B guidelines[25]. This was to better reflect and highlight the two main pathological processes of chronic infection and chronic hepatitis, in particular taking into account the presence of hepatitis B e antigen (HBeAg), HBV DNA levels, alanine aminotransferase (ALT) values and the presence or absence of liver inflammation. The new definition of phases highlights the increased risk of advancing liver disease in both chronic hepatitis phases - even in HBeAg negative patients - where there is elevated HBV DNA levels and/or elevated ALT, removing the somewhat misleading term "inactive carrier". These changes in nomenclature have now been widely adopted[24].

Multiple societies now provide guidance on when to initiate treatment. Viral resistance to treatment is a problem which has now been largely overcome. The nucleos(t)ide analogues (NAs) entecavir (ETV), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide are recommended as first-line treatment in both American and European HBV guidelines[25,26]. These agents show high rates of viral suppression and high genetic barriers to resistance[27,28] and have largely replaced lamivudine (LAM) with which resistance was problematic and common. Following treatment with LAM for 1 year, 14%-32% of patients developed resistance, increasing to over 80% after 4 years[27]. Those who develop resistance to LAM and are switched to ETV are more likely to subsequently develop resistance to ETV, with resistance rates of up to 50% after 5 years of treatment compared to only 1.2% of patients developing resistance with ETV where LAM has not been previously used[27]. TDF monotherapy has been shown to be effective in patients who have previously experienced treatment failure due to LAM resistance[29] and although there have been cases reported of reduced efficacy of tenofovir, there have been very few reported cases of resistance.

Problems remaining to be solved

There remain a number of challenges in the diagnosis and management of patients with chronic hepatitis B infection - Figure 1.

Diagnosing and linking infected patients to care programmes: A significant proportion of infected persons have not been identified; current estimates suggests that only 10.5% of infected individuals have been diagnosed and only 5% of those eligible for treatment for chronic HBV infection are receiving treatment[30]. A large systemic review found that 10% of people (26 million) with HBV infection might need urgent treatment due to cirrhosis and 12%-25% of patients would also be eligible for treatment according to different international guidelines[30]. Many countries do not have the infrastructure to deliver widespread testing, vaccination or treatment; this is particularly true in low-middle income countries where resources are limited. Detailed discussion on the challenges of such health inequalities are beyond the scope of this review. The approach to up-scaling diagnostic testing needs to vary according to the target population. In the United Kingdom and other developed countries, the majority of individuals with undiagnosed hepatitis B infection are born in countries with intermediate or high prevalence rates. Identifying these individuals may increase diagnosis rates. Case finding in high-risk groups is effective; in North-East England, individuals from the British-Chinese and South Asian communities were invited to education and screening (*via* dry blood spot testing) sessions in local community centres[31]. The prevalence of hepatitis B surface antigen (HBsAg) positivity was 4.6%, which is above the 2% screening threshold recommended by the Centers for Disease Control and Prevention[31]. Another study looked at the cost-effectiveness of a one-time opt out case-finding approach in a primary care setting in the United Kingdom migrant population. This approach was deemed very likely to be cost effective amongst migrant populations with HBsAg prevalence ≥ 1%[32].

New point of care (POC) tests are also becoming available, making diagnosing infection easier and quicker. For example, the Determine HBsAg 2 test provides a HBsAg result in 15 min with high sensitivity and specificity[33]. POC tests allow testing and diagnosis to move out of established health care settings and may be of particular utility in resource poor settings and high-risk communities.

Increasing testing and subsequent diagnosis rates relies on public engagement to break down stereotypes and address stigma, improved interactions with health care services and addressing health inequalities arising from poverty and language barriers [34,35]. Collaboration and integration with other successful public health programs such as human immunodeficiency virus (HIV) services is also likely to be effective.

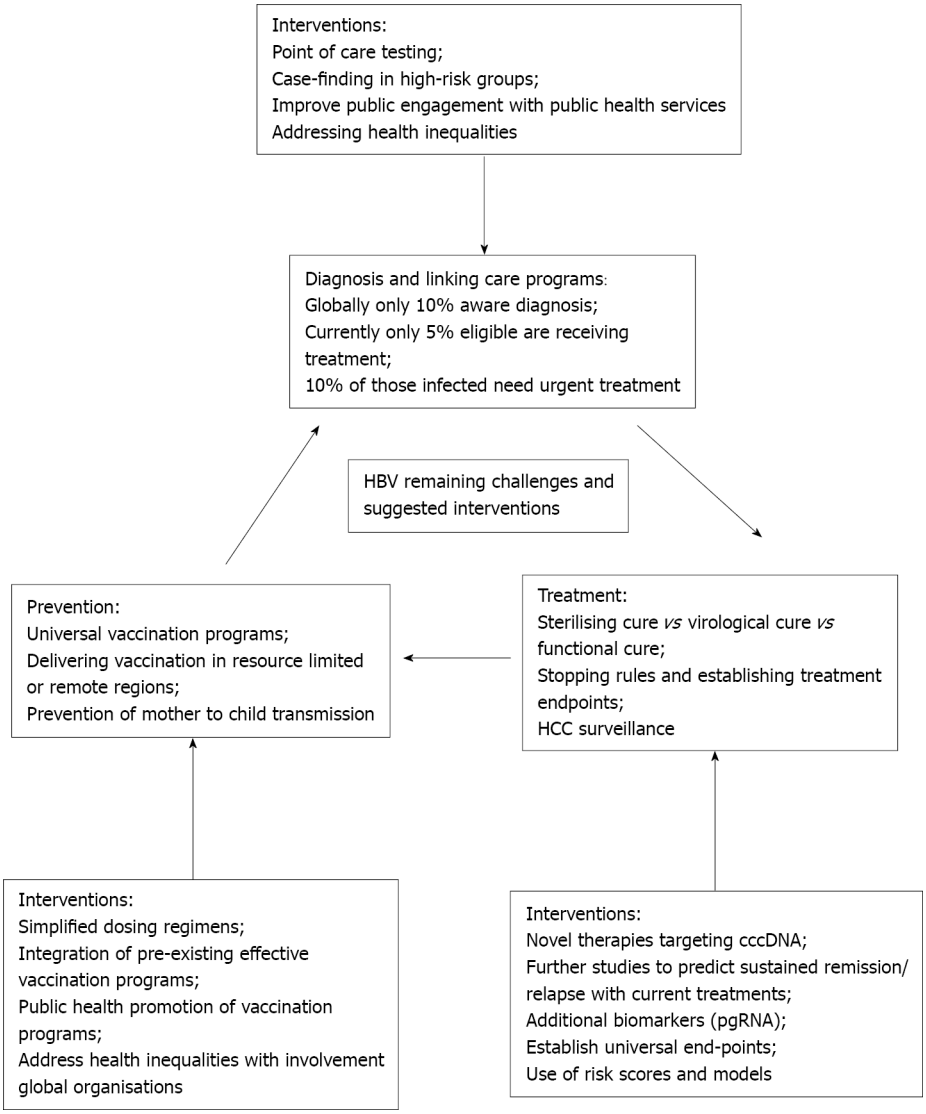


Figure 1 Remaining challenges in hepatitis B virus infection. HBV: Hepatitis B virus; cccDNA: Closed circular DNA; HCC: Hepatocellular carcinoma; pgRNA: Pregenomic RNA.

Defining cure: A 'cure' for HBV might be considered as one where the virus is completely eliminated [undetectable HBsAg, HBeAg, HBV DNA and hepatic co-valently closed circular DNA (cccDNA)] and where any (risk of) associated liver disease is also removed[36]. Consensus on definitions of cure remain contentious and as there is no current or upcoming treatment to achieve the 'holy grail' described above, there is reticence in how the word 'cure' is used. However, this is a key aspect of clinical care and research, therefore a globally accepted definition of cure needs to be obtained.

The term 'sterilising cure' (complete eradication of the virus) has been replaced with 'functional cure'. Functional cure is currently defined as sustained HBsAg loss, undetectable HBV DNA, with or without seroconversion to hepatitis B surface antibody, following a finite course of treatment[25] and it occurs in 1% of chronically infected patients annually[37]. However, HBV genomes can persist in the liver even if HBsAg is undetectable questioning the true value of achieving a functional cure. A 'partial functional cure' is considered an intermediate goal of therapy and signifies detectable HBsAg but persistent undetectable HBV DNA 6 mo post-treatment. Virologic cure is essentially 'halting' all forms of HBV replication, however difficulties with obtaining virologic cure remain due to the persistence of cccDNA in hepatocytes. To obtain virologic cure, treatments inhibiting both cccDNA and viral replication are required[38].

An agreed definition of cure remains elusive, however with clearly defining treatment endpoints and new therapies targeting different aspects of the HBV life

cycle, virologic cure may be achievable in the future.

Striving for prevention rather than cure: To prevent HBV infection, there needs to be a focus on improving vaccination strategies. Barriers to HBV vaccination, particularly in resource limited or remote regions, can be attributed to inadequate resources to acquire vaccinations, current dosing regimens, insufficient trained health staff for administration of the vaccine and lack of facilities to keep vaccinations between 2-8 °C. One study of a two-dose regime of HBsAg-1018 (containing HBsAg plus a toll-like receptor 9 agonist adjuvant) demonstrated a higher seroprotection rate at one year compared with the standard three dose regimen[39]. Simplified regimens with fewer doses over a shorter time period (HBsAg-1018 given at 0 and 4 wk) are likely to be associated with increased uptake[39]. Many countries have now instituted effective COVID-19 vaccination programmes, and similar systems could be used to roll out simplified HBV vaccination regimens.

Preventing mother to child (vertical) transmission of hepatitis B is vital if global elimination is to be achieved[40,41]. High maternal viral load is the greatest risk factor for mother to child transmission; HBeAg positivity also increases risk[41]. In resource poor settings the WHO-recommended vaccine strategy may be difficult to deliver, and diagnostic assays for HBV testing may not be readily available. A potential strategy in these settings is POC testing to establish HBeAg status, followed by empirical treatment with tenofovir in the 3rd trimester in those who are HBeAg positive to reduce viral load and the risk of perinatal transmission[42], however such diagnostic assays are not readily available and remain costly.

Defining 'stopping rules' for HBeAg negative patients treated with NAs: Where seroconversion of HBeAg occurs, 67%-85% of patients have a sustained inactive state (HBeAg negative chronic infection); this is particularly the case where seroconversion occurs below the age of 30 years and where a low or undetectable HBV DNA level has been maintained[43]. However, given significant relapse rates it remains controversial as to whether NA treatment can be stopped after HBeAg loss. A HBeAg negative state is associated with higher rates of regression of fibrosis but some patients will develop HBeAg negative hepatitis, the risk of which increases with time (22% at 10 years) and increases the risk of progression to advanced liver disease[44].

Given the low rate of clearance of HBsAg, HBeAg seroconversion is considered as a potential endpoint of treatment, where undetectable HBV DNA is achieved on three separate occasions in a 6[25] or 12-mo[26] period. If treatment is stopped at this endpoint, 50% will undergo HBeAg reversion requiring treatment with NAs to restart; close biochemical monitoring is therefore required. There is evidence to suggest that longer treatment with NAs results in a higher chance of persistent remission, with viral remission for 24-mo on NAs offering the most likely chance of sustained remission[45].

Therefore, currently there is no universal stopping rule. In real-world practice, many different factors are taken in to consideration when making the decision to stop treatment with NAs, including the stage of fibrosis and family history of hepatocellular carcinoma (HCC). Further studies are needed to more clearly define the predictors of sustained remission and/or relapse to guide stopping decisions.

Establishing treatment endpoints - aiming for viral suppression *vs* cure: Currently, long-term suppression of HBV DNA levels is the main endpoint of treatment (+/- HBeAg loss in HBeAg positive patients). It remains a subject of debate as to whether the endpoint of treatment should be viral suppression, functional cure, partial functional cure or virologic cure. The ideal goal however would be virologic cure. In 2019 the joint EASL-American Association for the Study of Liver Diseases HBV treatment endpoints conference agreed that a "functional cure" should be the primary endpoint of phase III trials; sustained HBsAg loss in more than 30% of patients was accepted as an acceptable rate of response in phase III trials[38]. The endpoint for trials may not be the same as the endpoint for real world clinical practice however.

Biomarkers continue to be developed and may prove useful in defining future treatment endpoints. These biomarkers are likely to be used in conjunction with currently utilised clinical markers. The development of hepatitis B core-related antigen (HBcrAg) as a potential serological marker for cccDNA levels may identify patients who could discontinue NA therapy, those at risk of HCC development or of recurrence following treatment[28,46]. Pregenomic RNA may be a novel marker of viral replication; evidence is emerging that this may provide an earlier predictor for HBeAg seroconversion for those patients on NAs (an important indicator for partial immune response) and may help guide future treatment in those not achieving HBeAg seroconversion[47].

Establishing a universally accepted endpoint of treatment along with biomarkers to help predict or confirm the achievement of this endpoint would be an important advance in the treatment of chronic HBV infection.

Risk of HCC and surveillance in patients on long term NAs: Chronic HBV infection is a leading cause of HCC; it is responsible for around 25% of liver cancer cases in developed countries and up to 60% of cases in developing countries[48]. NA therapy has been reported to decrease incidence of HCC[49,50]. While HBsAg loss after the development of advanced fibrosis minimizes the risk of the development of HCC, it does not negate it completely[49]. A number of factors are taken into consideration when deciding which patient to survey for HCC including disease phase, age, ethnicity and family history of HCC[49]; international guidelines do not agree on the populations for surveillance however, promoting inequalities in care.

In those on NA therapy, risk scores such as the REACH-B score[51] or PAGE-B score[52] are used to identify patients who would benefit from HCC surveillance. The REACH-B scoring system was developed in a cohort of Asian patients with chronic HBV infection who were treatment naïve; no patients with cirrhosis were included in the development of this score[51]. This score does not offer good predictability in Caucasian patients with chronic HBV infection[53]. The modified REACH-B score substituted HBV DNA levels for the liver stiffness value which increased its accuracy [54]. The PAGE-B score was developed for use in Caucasian populations receiving tenofovir or ETV. A modified PAGE-B score (addition of serum albumin) has recently been tested in Asian patients on NA therapy, with an area under the receiver operating characteristic curve of 0.82[53]. The PAGE-B score is also predictive of HCC development in untreated patients[52].

Quantitative HBsAg and HBcrAg have been proposed as new biomarkers for HCC risk which might influence patient selection for HCC surveillance[55]. Risk models incorporating these biomarkers would be an advance in the field of HBV. New models could also incorporate other novel markers such as specific HBV mutations, presence of the metabolic syndrome and HBV genotype.

Identifying new treatments with finite duration and high cure rates: Most patients with chronic HBV currently require lifelong therapy, achieving viral suppression rather than cure[25,26]. To achieve cure, combinations of therapy targeting different aspects of the HBV lifecycle are likely to be required including inhibition of cccDNA and viral replication[38].

A number of new treatments are being investigated for HBV and these are aiming to achieve clearance of HBsAg rather than just suppressing HBV DNA[36]. A detailed description of these treatments is beyond the scope of this review, but these include the development of new NAs (besifovir and metacavir), cccDNA silencers (*e.g.*, lymphotoxin beta receptor agonist) and HBV entry inhibitors (Myrcludex B)[28,38,56]. There may also be a role for immunomodulatory therapies such as toll-like receptor agonists (acting *via* activating the innate immune response), check point inhibitors (helping to restore T-cell dysfunction) or therapeutic vaccines such as TherVacB[56, 57]. Gene editing strategies and RNA interference may be other potential treatment strategies[56]. Where eligible, patients should be considered for entry into clinical trials of novel therapies.

Hepatitis D virus

The current burden of hepatitis D virus (HDV) infection is unknown; estimates from a recent meta-analysis vary considerably, ranging from 12 million to 72 million individuals infected with HDV worldwide[58]. There is geographical variation in the prevalence of HDV infection. A recent systematic review and meta-analysis estimated anti-HDV prevalence to be 4.5% amongst HBsAg positive individuals globally with rates lower in Europe (3.0%) compared with Africa (5.97%)[58-60]. However, other meta-analysis estimates differ, demonstrating higher seroprevalence amongst HBsAg positive individuals worldwide (10.58%-13.02%) and within Europe (13.81%). Such differences are likely due to variation in modelling strategies and highlight the difficulties in truly identifying the burden of HDV[58-60]. Issues and challenges remaining in the field of HDV infection include identification of infected individuals, effective treatments, treatment endpoints and prevention.

Problems remaining to be solved

Identification of infected patients: A positive HDV antibody should be accompanied by detectable serum HDV RNA to detect active infection. However, some guidelines do not explicitly make recommendations for HDV testing and therefore many patients

who are HBsAg positive are not tested for HDV. One study looking at clinic-led anti-HDV testing identified that only 40% of HBV patients were tested[61]. The same study looked at a different centre offering reflex laboratory testing and found that 99.4% of first HBsAg positive samples were tested for anti-HDV. This is a potentially reliable approach to increasing detection of patients with HDV infection, as all patients who are newly diagnosed with HBsAg positivity should be tested for serological evidence of HDV infection.

There is an epidemiological association between anti-HDV seroprevalence and PWIDs, commercial sex workers, MSM and recipients of haemodialysis[58,62]. Suggested patient groups who should be prioritised for screening for HDV include: Patients who are HBsAg positive, patients with HIV, PWIDs, MSM and migrants from highly endemic regions.

Treatment for HDV infection: Pegylated-interferon **(**PEG-IFN) is the only treatment proven to have antiviral efficacy against chronic HDV infection, however viral suppression rates with PEG-IFN are poor in HDV infection and the adverse effects of PEG-IFN therapy are well described[62,63]. Extended duration of treatment has not been associated with a consistent or significant increase in efficacy, and the addition of NAs does not improve efficacy. New treatments are urgently required; therapies currently being evaluated include HBV/HDV entry inhibitors (Myrcludex B), virion secretion inhibitors (REP 2139) and inhibitors of the prenylation of the large HDV antigen (lonafarnib)[63]. Patients with HBV/HDV co-infection should be considered for entry into clinical trials. Ultimately, global prevention of HBV infection would be the most effective means of treating HDV infection.

Establishing treatment endpoints: Unfortunately, endpoints for HDV treatment and indicators of response to treatment have not been well established[38]. Cure may not be feasible. ALT normalization, changes in HDV RNA and qHBsAg are markers of response to treatment. Barriers to establishing treatment endpoints include lack of widespread availability of HDV diagnostics and lack of standardization of HDV RNA assays. Composite endpoints are likely to be more useful than singular end-points.

HEPATITIS C VIRUS

Perhaps the greatest advances in our understanding of virology and development of treatment strategies over the past decade have occurred in relation to hepatitis C virus (HCV) infection. Despite these advances a number of challenges remain, including targeting difficult to reach populations and expanding HCV testing and treatment programmes in resource poor countries. Addressing these areas will be critical if global elimination of HCV is to be achieved by 2030.

Advances in past decade and problems now solved

Treatment and cure: HCV treatment has evolved rapidly in the last 10 years, with the emergence of direct acting antiviral (DAA) regimens. These drugs are very well tolerated and highly effective in achieving sustained virologic response (SVR), even in patients who were previously considered 'hard to treat' or in whom interferon-based treatment was contraindicated. As a result, antiviral treatment with DAAs is recommended in all patients with active HCV infection[64] and elimination of HCV is an achievable goal if these drugs can be made widely available worldwide.

In 2011 the first protease-inhibitors (telaprevir and boceprevir) were approved for use in HCV infected individuals in combination with pegylated-interferon and ribavirin, but whilst SVR rates improved so did the frequency of side effects[65]. This was quickly followed by the approval of the first interferon-free regimens for the treatment of genotype 1 HCV infection in 2014, followed by the first pangenotypic regimen, sofosbuvir-velpatasvir, in 2016[66]. Pangenotypic regimens are advantageous because they remove the need for genotype testing prior to the commencement of treatment which simplifies treatment regimens, thus reducing the frequency of patients dropping out before they start antiviral treatment.

Presently, the availability of safe and highly effective DAA regimens supports a strategy of treating all individuals with chronic HCV infection over the age of 12, irrespective of the stage of disease[67]. Current regimens offer a number of advantages over previous interferon-containing regimens including much greater efficacy, few side-effects, oral once daily dosing and shorter duration of treatment. For current DAA regimes, SVR rates (undetectable HCV RNA at 12 or 24 wk after treatment) well exceed 90% for most patient cohorts, compared with approximately 50% of patients

treated with PEG-interferon and ribavirin. Patients with chronic kidney disease (including dialysis-dependent patients) and cirrhosis were previously considered difficult to treat but now have similar SVRs when treated with DAAs to those without chronic kidney disease and cirrhosis[68,69].

Significant improvements in SVR rates with DAAs has translated into a reduction in morbidity and mortality rates in patients with HCV. A systemic review and meta-analysis concluded that there was an 87% reduction in the incidence of HCC and a 75% reduction in all-cause mortality in those who achieved SVR when compared with those who did not[70]. By 2019 in the United Kingdom, the incidence of HCV-related end stage liver disease and HCC had fallen by 24% following the introduction of DAAs and the associated increase in the number of patients completing treatment. In Scotland, new presentations of HCV-related decompensated cirrhosis decreased by 51% in the DAA area with an estimated avoidance of 330 cases of decompensated cirrhosis[73].

Problems remaining to be solved

Prevention: The ideal preventative treatment for HCV would be a vaccine. However, development of an HCV vaccine has been challenging due to the genetic diversity of the virus, the virus' ability to avoid the host immune response and a lack of *in vitro* and *in vivo* models of infection[71]. Some progress has been made, and a recent trial of a vaccine regimen to prevent chronic HCV infection was safe and induced HCV-specific T-cell responses but it did not prevent chronic HCV infection in a cohort of patients with a recent history of intravenous drug use[72]. It is therefore unlikely that an available efficacious vaccine will be available in the short-term. Work to develop a vaccine is ongoing.

In the absence of a vaccine, improving harm reduction approaches for PWIDs is vital. Existing strategies include promotion of sterile injection equipment use through needle exchange programmes and opioid substitution therapy. These services are often poorly provided and under-utilized, but they have been shown to be highly cost-effective[73]. It is been estimated that eliminating non-sterile injection techniques could prevent 43% of incident HCV infections between 2018 and 2030[74].

Difficult to reach populations: Despite advances in the medical treatment of hepatitis C, global elimination is unlikely to be achieved unless all infected patients are identified and then complete their treatment regimen. A significant proportion of people with HCV infection are unaware of their diagnosis, and our ability to find these patients is becoming increasingly challenging. Previous work has shown that HCV testing is concentrated in areas with lower risk of infection[75], commonly settings where patients are either in recovery from previous drug use or ongoing drug use is more 'controlled'. Testing needs to be expanded among 'difficult to reach' populations, especially those who may be in a more 'chaotic' phase of their drug use and are not in contact with addiction or other medical services. This group can be challenging to find and engage, but approaches such as testing and treatment in homeless hostels and food kitchens can be effective[76]. Moreover, testing delivered by peers is an approach that can increase diagnosis and subsequent treatment in patients considered hard to reach. In the United Kingdom, the hepatitis C trust run a peer-to-peer education programme, in which peer educators with personal experience of HCV deliver workshops sharing the importance of testing and treatment[77]. This has increased testing numbers and reduced attrition, whilst providing valuable education.

One important area to target to increase testing and treatment is in the prison population. Prison populations have a high prevalence of HCV infection with many studies reporting an incidence > 10 times that of the general population[78]. Drug use prior to or during imprisonment is common, yet harm reduction methods such as access to clean injecting equipment is non-existent or inadequate in the majority of prisons. Opt-out screening for blood borne viruses (BBVs) is recommended in the EASL HCV guidelines for all prison inmates[79], but even where this is practiced rates of testing are suboptimal[78]. Opt-in testing is more commonly practiced but is a far less effective approach. BBV testing can be challenging, particularly in reception prisons (prisoners awaiting sentencing) because these typically have a very large throughput of inmates and periods of incarceration can be short. However, these challenges can be effectively overcome with investment and an organized approach to testing. Effective approaches to increasing testing for HCV and scaling up of treatment with DAAs can also be used as 'treatment as prevention'. This approach was practiced in an Australian prison population and led to a significant reduction in incidence of new HCV infections[80].

Another approach that could be considered to identify undiagnosed patients with HCV is a 'track and trace' approach by mapping the social networks of individuals with a history of injecting drug use and offering HCV testing to those in a group who may not have been tested. Whilst this may sound like a practical solution, one study showed that this was ineffective in real world clinical practice with only one participant coming forward for testing[81]. Further work is needed to determine whether this approach could be refined to increase its efficacy, particularly since people are now more aware of 'track and trace' programmes as a result of the COVID-19 pandemic.

Attrition: Increasing detection rates will only help in the strive for global elimination if these are translated into increased treatment rates. An analysis of two large national laboratory databases from 2013 to 2016 found that 89.4% of patients diagnosed with chronic HCV infection did not receive a prescription for antiviral therapy[82]. In Spain, 49.8% of those with a positive anti-HCV result were not then linked into specialist care [75]. One reason for this is that care pathways have been unnecessarily complex including multiple investigations prior to treatment, which leads to patients frequently being lost to follow up and never completing treatment. Attrition appears to occur early in the treatment cascade; in one study 57.3% of patients dropped off prior to having liver enzymes checked[83]. With the advent of pangenotypic regimens and simple non-invasive fibrosis scores [*e.g.*, fibrosis-4 (FIB-4) and aminotransferase-platelet ratio index] pathways can effectively be simplified, which is likely to reduce attrition. An ideal pathway is shown in Figure 2.

Moving care delivery out of hospital settings may improve attrition rates. One study from the North-East of England found that distance from a HCV treatment service was a major predictor of patients not commencing antivirals[84]. DAAs can be effectively delivered in non-hospital settings, increasing access to treatment. A cluster-randomized trial showed that pharmacist delivered treatment in patients on opiate substitution therapy was more effective than conventionally delivered HCV therapy with more patients initiating and completing treatment, and achieving SVR[85]. Other examples of successful non-traditional HCV services have been delivered in primary care, nurse led community clinics, addiction services and homeless hostels[86]. Empowering addiction workers and those working with the homeless to become involved in the care cascade is also likely to improve attrition rates.

Reinfections: Re-infection with HCV after SVR, detected by the presence of HCV RNA rather than HCV antibodies, is largely related to an individuals' ongoing high risk behavior, inadequate harm reduction knowledge and/or lack of availability of clean injecting equipment. The true rate of reinfection is not known and is likely to vary significantly depending on the population studied. Individuals who continue to actively inject drugs after treatment have the highest rates. Very high rates of reinfection (up to 40%) have been seen in some high-risk groups, but other studies have reported lower rates[87]. Better access to harm reduction methods is vital to reduce reinfection rates. In addition, PWIDs should be tested at least annually for HCV RNA if they have ongoing high-risk behavior to identify reinfections. It is critical that they are offered re-treatment to try and reduce the risk of onward transmission of the infection[79].

Long term impact of hepatitis C infection: HCV infection is associated with multiple extrahepatic complications including increased risk of autoimmune disorders, cryoglobulinaemia and lymphoma. In addition, there is increased risk of type II diabetes, cardiovascular disease, chronic fatigue and psychological morbidity. Many of these comorbidities persist following SVR and one study found that nearly all patients have at least one co-morbidity that remains long-term[88].

Despite individuals with HCV having a significantly increased risk of cardiovascular disease, few are actively treated to reduce their cardiovascular risk[89]. Moreover, even though quality of life improves following successful antiviral treatment, this remains significantly worse than the general population[89]. This probably relates to high rates of mental health disorders, unhealthy alcohol consumption, ongoing drug use, deprivation, type II diabetes and the metabolic syndrome. Participation in physical activity in individuals with HCV is associated with improved quality of life[90]. Taking a more holistic approach to the care of individuals with HCV rather than just focusing on treating the infection may help improve long-term outcomes and improve quality of life. Use of a holistic care bundle may help achieve this[89].

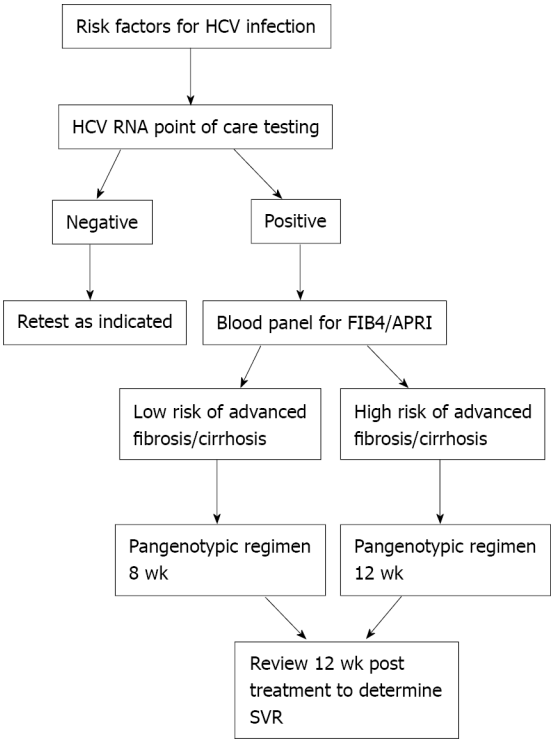


Figure 2 Proposed simplified pathway for hepatitis C virus diagnosis, staging and treatment. HCV: Hepatitis C virus; FIB-4: Fibrosis-4; APRI: Aminotransferase-platelet ratio index; SVR: Sustained virologic response.

HCC surveillance in non-cirrhotic patients: The risk of development of HCC in individuals with HCV-related cirrhosis falls following SVR, but remains approximately 2% per year and as a result, surveillance is recommended for these individuals [91]. HCC may also occur in patients with advanced fibrosis, but at a lower rate than in those with cirrhosis and it remains uncertain whether HCC surveillance is clinically effective and cost effective in this group. This is further complicated by the fact that many patients are staged using transient elastography and relevant cut-offs to identify those who are likely to benefit from HCC surveillance have not been defined. There is a clear cut need to develop better models to predict the development of HCC in individuals following SVR. International societies have different recommendations regarding HCC surveillance in those achieving SVR which reflects the overall uncertainty - Table 1.

There have been some recent studies that have attempted to more clearly define patients who would benefit from HCC surveillance post SVR[90,91]. One study developed a model to predict patients with advanced fibrosis who have a low risk of HCC and may therefore not benefit from surveillance. The model used a combination of baseline and dynamic changes in liver stiffness measurement, FIB-4 score and serum albumin after SVR and identified that nearly 20% of their cohort of patients with compensated advanced fibrosis had a very low risk of developing HCC[90]. Dynamic assessment of the FIB-4 score in isolation may also predict the risk of development of HCC after SVR[98]. In one study, no patients with a FIB-4 < 1.45 after SVR developed HCC[92]. A number of studies are underway with the aim of developing better predictive models for HCC using clinical parameters and novel biomarkers.

Increased use of hepatitis C positive donor organs: The advent of safe and highly effective DAAs for HCV infection has increased the potential to use HCV-positive organs even when the donor is viraemic, expanding the donor pool[93]. HCV positive (HCV RNA +) donor organs universally transmit HCV to the recipient[94] so prior to the widespread availability of DAAs use of these organs was restricted to those who already had HCV viraemia. However, given the efficacy of DAAs it is now possible to transplant HCV RNA + organs in to HCV negative recipients and then treat the HCV infection in the recipient.

In 2019, Kwong *et al*[95] assessed the outcomes from HCV treatment with DAAs in 10 non-viraemic patients who received HCV RNA + livers. Short-term outcomes were

Table 1 Recommendations for hepatocellular carcinoma surveillance in patients with hepatitis C virus achieving sustained virologic response

Guideline	Recommendation
EASL[114] 2020	Survey patients with advanced fibrosis (F3) or cirrhosis (F4)
AASLD[115]	Survey cirrhotic patients
Asia-Pacific[116]	Survey cirrhotic patients; Survey patients with any histologic stage of HCV with comorbidities, such as alcohol abuse and diabetes mellitus

HCV: Hepatitis C virus. AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver.

excellent with 100% achieving SVR at 12 wk post treatment. The practice of using HCV RNA + organs with subsequent DAA treatment is now routine in some countries around the world.

HEPATITIS E VIRUS

Hepatitis E virus (HEV) is the most common cause of acute hepatitis worldwide and carries a significant global burden of disease. HEV genotypes 1 and 2 account for approximately 20.1 million HEV infections, 3.4 million symptomatic cases, 70000 deaths, and 3000 stillbirths annually[96]. Our understanding of the impact of hepatitis E infection has advanced significantly over the past decade, with the recognition of chronic infection, risk of progression to cirrhosis, risk factors for transmission and treatment strategies. Despite these advances, there are problems that remain to be resolved.

Advances in past decades and problems now solved

There are now eight recognized genotypes of HEV. Genotypes 1-4 and 7 cause human infection. Genotypes 1 and 2 are obligate human pathogens transmitted by the faeco-oral route and cause both sporadic infection and large outbreaks. In the developed world, sporadic infections are mainly caused by genotype 3 infection.

Transmission of HEV infection

Ingestion of raw or under-cooked meat (particularly pork products), shellfish and contaminated fruits is a significant risk factor for locally-acquired infection in the Western world. Genotype 3 and 4 HEV infection can be transmitted *via* transfusion of infected blood products and solid organ transplantation, and may have a significant clinical impact upon immunosuppressed individuals. A French study looked at 23 cases of reported transfusion related HEV infections in France between 2006-2016. It reported that 14 of these cases, all of whom were immunosuppressed, went on to develop chronic HEV infection[97]. The United Kingdom introduced a universal screening policy for blood products in 2017 and also screens deceased and live organ donors for HEV RNA[98]. Other countries have a more selective strategy and only screen blood products intended for high-risk patients[99]. Universal screening has been shown to be more cost effective than selective screening if the incidence of HEV infection is above 1 in 10000 blood donations[100]. Sexual transmission in MSM has also been reported more recently[101].

Chronic infection and risk of cirrhosis

Prior to 2008, HEV was recognized to cause an acute, self-limiting illness. Genotype 3 HEV was first reported to cause chronic infection in 2008 and chronic infection has now been reported in immunocompromised individuals including solid organ transplant (SOT) recipients, patients receiving chemotherapy for haematological malignancies, HIV-1 infected patients and patients receiving immunomodulating drugs. In immunocompromised patients, the detection of HEV RNA in plasma or stool after 3 mo is defined as chronic infection[102]. Progression to cirrhosis in those with chronic hepatitis E infection occurs in 10%-15% and can occur rapidly, within 2-3 years [103]. In a study of 85 patients with chronic HEV infection in 17 transplant centres across Europe and North America, almost 66% of transplant recipients who contracted HEV developed chronic infection and 10% progressed to cirrhosis[103,104]. Chronic infection and the risk of cirrhosis is not seen with genotype 1 or 2 infection.

Treatment of chronic infection

Most published data regarding treatment of chronic HEV infection are from case series and reports in SOT recipients[105]. Reducing immunosuppression dose by around 30% has been shown to be effective in clearing HEV in around one third of patients [106]. Both PEGylated interferon and ribavirin are effective in treating chronic HEV infection. Interferon increases the risk of organ rejection in transplant recipients and therefore ribavirin monotherapy is the preferred option[107]. A systematic review has shown that 64% of patients were HEV RNA negative at 6 mo after the end of treatment with ribavirin monotherapy[108]. The optimal dose and duration of treatment is still to be determined but 3 mo courses have been used most commonly[107]. A multi-centre case series of 59 transplant recipients infected with HEV showed that ribavirin monotherapy, at a median dose of 600 mg/d for 3 mo achieved SVR in 78% of cases [107].

Problems remaining to be solved

Non-response to ribavirin: The main problem to be solved in relation to chronic HEV infection is how to manage non-response to ribavirin. Sofosbuvir has been proposed as an alternative agent to treat chronic HEV infection. It has shown promise in inhibiting HEV replication *in vitro*[109] but it had a negligible effect on improving viraemia in a case report[110]. A later study of sofosbuvir monotherapy in nine patients demonstrated a modest reduction in viral load but viral elimination was not achieved[111]. Convalescent plasma has also been trialed in a patient with persistent hepatitis E infection, and showed no effect on HEV RNA levels.

We also need to understand the relevance of HEV mutations and their effect on ribavirin resistance. Mutations have been identified in ribavirin non-responders but their impact on the treatment of these and other individuals has yet to be established. For example, the G1634R mutation does not lead to absolute ribavirin resistance and does not appear to compromise the response to a second course of treatment with ribavirin[112]. New treatments are ultimately required for those who fail treatment with ribavirin.

IMPACT OF COVID-19 ON VIRAL ELIMINATION

The COVID-19 pandemic has compromised efforts to progress towards the WHO goal of elimination of viral hepatitis. This impact of the pandemic is likely to be felt for years to come and during the initial peaks has resulted in delays in diagnosis and treatment, and reduced access to harm reduction services. In April 2020 in the United Kingdom, new diagnoses of HCV were down 85% and new treatment initiations had also fallen by 63% compared with the year prior[113]. Although there has been some recovery, pre-COVID 19 levels of testing and treatment have not yet been reached. Funding and resources have also been re-allocated to fighting the COVID-19 pandemic. In addition to the impact on global elimination, the COVID-19 pandemic has significantly impacted upon the provision of HCC surveillance programmes for patients with viral hepatitis.

However, during the pandemic many new ways of working (such as telemedicine) and care cascades have been adopted, which may in fact positively impact upon the delivery of viral hepatitis services in the years to come. For example, in some centres patients have been commenced on HCV treatment remotely using telemedicine (personal communication). The vaccination programmes and 'track and trace' systems set up during the COVID-19 pandemic could be extrapolated to viral hepatitis to improve service delivery.

SUGGESTED PUBLIC HEALTH AND RESEARCH PRIORITIES FOR THE NEXT DECADE

The global hepatology community is well placed to set public health and research priorities in viral hepatitis for the forthcoming decade, striving towards global elimination and reduced health care burden. Potential priorities for each individual virus are proposed in Table 2.

Table 2 Public health and research priorities for the next decade

Virus	Public health priorities	Research priorities
Hepatitis A	Increased vaccination of high-risk individuals; Improved sanitation and vaccination in camps for displaced persons	Medical treatments for those with acute liver failure
Hepatitis B	Increase uptake of vaccination; Identifying undiagnosed individuals; Linkage to care	Establishing treatment end-points; Identifying curative treatment
Hepatitis C	Microelimination; Reducing re-infection rates; Identifying undiagnosed individuals; Harm reduction	Vaccination; Confirming most effective HCC surveillance strategies
Hepatitis D	Identification of infected individuals; Clarifying current disease burden of HDV	Novel therapies
Hepatitis E	Increased screening of blood products/change in donor policies; Educating immunosuppressed patients of risk of food-borne transmission; Further understanding of sources of infection	RCT to confirm optimal dose and duration of ribavirin therapy; Novel treatments; Vaccination; Greater understanding of genetic mutations

HDV: Hepatitis D virus; HCC: Hepatocellular carcinoma; RCT: Randomized controlled trial.

CONCLUSION

Significant advances have occurred in the field of viral hepatitis over the past decade, particularly in relation to the treatment and cure of hepatitis C. Over the next decade – as we strive towards global elimination of viral hepatitis – the gastroenterology and hepatology community must focus on identifying the undiagnosed and engaging these individuals in to treatment programmes whilst continuing to develop novel treatments with the ultimate aim of cure.

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MINIREVIEWS

Risk of hepatocellular carcinoma after hepatitis C virus cure

Maria Alejandra Luna-Cuadros, Hao-Wei Chen, Hira Hanif, Mukarram Jamat Ali, Muzammil Muhammad Khan, Daryl Tan-Yeung Lau

ORCID number: Maria Alejandra Luna-Cuadros 0000-0001-9259-2565; Hao-Wei Chen 0000-0003-0279-4214; Hira Hanif 0000-0002-1888-0056; Mukarram Jamat Ali 0000-0003-3313-9933; Muzammil Muhammad Khan 0000-0003-1324-3637; Daryl Tan-Yeung Lau 0000-0003-4139-1987.

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Maria Alejandra Luna-Cuadros, Hao-Wei Chen, Hira Hanif, Mukarram Jamat Ali, Muzammil Muhammad Khan, Daryl Tan-Yeung Lau, Liver Center, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Corresponding author: Daryl Tan-Yeung Lau, MD, MSc, Associate Professor, Liver Center, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, 110 Francis Street, Suite 4A, Boston, MA 02215, United States.
dlau@bidmc.harvard.edu

Abstract

Hepatitis C virus (HCV) is a significant cause of hepatocellular carcinoma (HCC). The direct-acting antivirals marked a new era of HCV therapy and are associated with greater than 95% cure rate. Successful treatment of chronic hepatitis C greatly reduces the risk of HCC. A proportion of patients, especially those with pre-existing cirrhosis, remain at risk for HCC despite sustained virologic response (SVR). Diabetes mellitus, hepatic steatosis, alcohol consumption and lack of fibrosis regression are associated with risks of HCC after HCV cure. Noninvasive modalities such as aspartate aminotransferase to platelet ratio index and fibrosis-4 index and transient elastography have been used to monitor hepatic fibrosis. More recently, various fibrosis scores have been combined with clinical parameters and other novel biomarkers to predict risks of HCC for patients who achieved SVR. These models still need to be validated and standardized prior to applying to routine clinical care.

Key Words: Hepatitis C virus cure; Hepatocellular carcinoma; Hepatocellular carcinoma risk models; Fibrosis markers; Transient elastography

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Core Tip: Direct-acting antivirals (DAA) therapy has revolutionized the treatment for chronic hepatitis C. However, the development of hepatocellular carcinoma (HCC) after achieving DAA-induced sustained virologic response remains a significant concern, especially those with advanced fibrosis. It is critically important to monitor hepatic fibrosis and continue HCC surveillance for patients with pre-existing cirrhosis. Lack of hepatic regression and several comorbid conditions are associated with HCC

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risks. Some promising models for predicting HCC risks after hepatitis C virus cure are in development.

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INTRODUCTION

Hepatitis C virus (HCV) is a global health issue affecting 160-170 million people worldwide[1]. According to recent National Health and Nutrition Examination Survey data, there are approximately 2.4 million people with chronic hepatitis C (CHC) in the United States[2]. There are 6 major genotypes of HCV[3]. Globally, G1 is most common accounting for 49.1% of all infections among adults, followed by G3 (17.9%), G4 (16.8%), G2 (11.0%), G5 (2.0%) and G6 (1.4%)[3]. There are significant geographic variations in the 6 HCV genotypes (Table 1). G1 is the predominant HCV genotype, for example, in North America, Europe, Caribbean and Latin America. G4 is most common in North Africa especially Egypt and the Middle East. The high prevalence of G3 in Asia is largely contributed by South Asia in particular India and Pakistan[3].

HCV-related hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, accounting for 85%-90% of primary liver cancers[4]. Advanced stage liver fibrosis (Metavir stage F3) carries an increased risk of HCC, and patients with cirrhosis (Metavir stage F4) have an annual HCC incidence of approximately 4%[4]. With the advent of direct-acting antivirals (DAA) therapy, over 95% of the treated patients were able to achieve sustained virologic response (SVR) or HCV cure[5]. HCV cure reduces the HCC risk but those with preexisting cirrhosis remain at risk[6,7]. This review focused on the pathogenesis and risk factors of HCC after HCV cure, and the applications of noninvasive modalities and models to predict HCC.

NATURAL HISTORY OF HCV INFECTION

The transmission of HCV occurs mainly *via* blood with the majority due to unsafe injection use (intravenous drug use, healthcare workers in underdeveloped countries) and blood transfusion recipients before 1992[8]. Moreover, sexual transmission of HCV has significantly increased in human immunodeficiency virus-infected MSM in recent years[9,10].

After the virus transmission, HCV RNA reaches a detectable level in the serum in 7 to 21 d[11,12]. HCV RNA levels rise rapidly during acute infection but it generally takes 4-12 wk for the elevation of alanine aminotransferase (ALT) (indicative of hepatic injury) with an associated increase of serum bilirubin[13]. HCV itself is not cytolytic, but it generates potent innate and adaptive immune responses with cytotoxic cytokines production and hepatic injury[14]. Acute liver failure due to HCV is rare, but its incidence increases especially in patients with pre-existing chronic liver diseases[12].

Spontaneous eradication of HCV with recovery occurs only in only 15%-25% of patients with acute hepatitis C. The presence of homozygous rs12979860-C alleles in the interferon lambda gene, however, is associated with about 80% of spontaneous recovery[15,16].

CHC is defined as the persistence of HCV RNA six months after the initial infection. CHC can lead to progressive fibrosis, cirrhosis, end-stage liver disease and complicated with HCC (Figure 1). It is estimated that 20%-30% of patients with CHC will develop cirrhosis after a period of 20 years[17]. Monitoring the development of fibrosis over time can provide a more accurate progression to cirrhosis. A study of paired liver biopsies scored by the same pathologists suggested the time to develop cirrhosis from diagnosis is about 30 to 40 years[17].

Table 1 Regional prevalence of hepatitis C virus genotypes

Regions	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	G6 (%)	Mixed
Africa	26.3	23.7	6.3	28.1	12.2	-	3.4
North Africa/Middle East	27.3	0.8	6.3	65.3	0.3	-	-
North America	66.3	13.1	15.7	4.3	-	0.6	-
Caribbean	83	7.2	2.1	0.6	-	0.1	7.0
Central Latin America	74.6	21.6	3.3	0.1	0.1	-	0.3
Central Asia	70.4	8.6	19.6	-	-	-	1.4
South Asia	15.5	1.9	66.7	3.7	0.1	0.5	11.6
Europe	64.4	5.5	25.5	3.7	0.1	0.1	0.7
Australasia	55.0	6.5	36.0	1.2	-	1.3	-

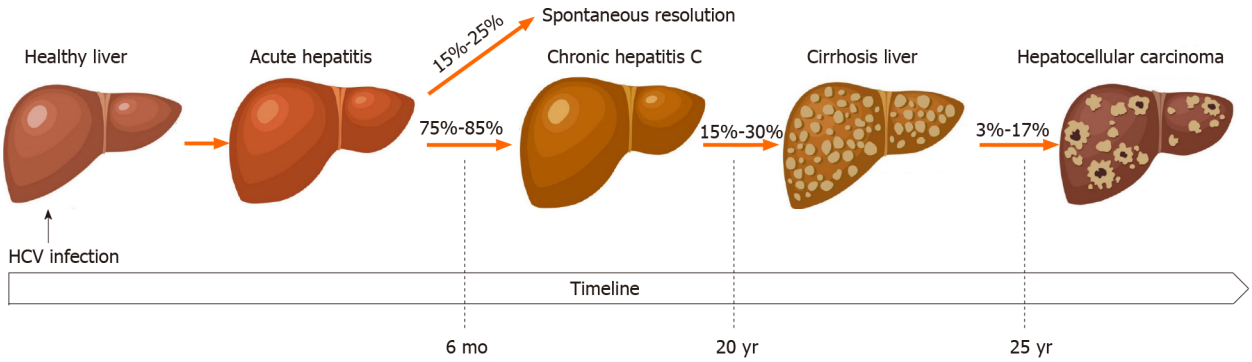


Figure 1 Natural history of chronic hepatitis. HCV: Hepatitis C virus.

After the progression to cirrhosis, patients are at increased risk of decompensated liver disease with associated complications such as ascites, spontaneous bacterial peritonitis, variceal bleeding and hepatic encephalopathy. The development of any of these complications is an indicator of increased risk of death or need for liver transplantation. Among patients with compensated cirrhosis, the 5-year and 10-year survival was 85%-91% and 60%-79% respectively[18]. The rate of clinical decompensation was 2%-5% per year and incidence of HCC was 1%-4% in these patients[18]. Generally, the risk for HCC and death increases significantly once decompensation develops[18].

HCV CURE

Treatment for HCV has revolutionized in the last decade. Before 2011, interferon was the mainstay of the therapy for HCV. Pegylated interferon combined with ribavirin had a success rate of 70% and 80% for genotype 2 and 3, respectively. However, the efficacy of interferon in HCV genotype 1 was low at 10%-20% only[19]. The advent of DAA marked the new era of HCV cure (Table 2). Boceprevir (Victrelis®) and Telaprevir (Incivek®) were the first DAA agents approved for the treatment of genotype 1 HCV infection and multiple other regimens obtained approval in the ensuing years. Since 2016, there are three pangenotypic combination therapies against genotype 1 to 6 with potent efficacy.

HCV cure or SVR is characterized by the absence of detectable HCV RNA in the serum 12 wk after the completion of DAA therapy[20]. In a meta-analysis of 43 studies, the risk of relapse or reinfection in the low-risk patients was 0.95% [95% confidence interval (CI): 0.35%-1.69%] over a 5-year period. Among the high-risk populations, such as injecting drug users or prisoners, the reinfection rate increased to 10.67% (95%CI: 6.38%-15.66%) in 5 years[21].

Table 2 Current therapies for treatment of chronic hepatitis C

Year approved	FDA approved therapy	Genotype	Trade name
2011	PegIFN/RBV + Boceprevir	Genotype-1	Victrelis®
2011	Telaprevir + PegIFNα/RBV	Genotype-1	Incivek®
2013	Sofosbuvir + RBV or Sofosbuvir + PegIFNα/RBV	Genotype-1, 2, 3 and 4	Sovaldi®
2014	Ledipasvir + Sofosbuvirwith or without RBV	Genotype-1, 4, 5 and 6	Harvoni®
2015	Daclatasvir + Sofosbuvir with or without RBV	Genotype-1, and 3	Daklinza™ + Sovaldi®
2016	Grazoprevir + Elbasvir + RBV	Genotype-1, and 4	Zepatier™
2016	Velpatasvir + Sofosbuvir	Genotype 1 to 6	Epclusa®
2017	Glecaprevir + Pibrentasvir	Genotype 1 to 6	Mavyret™
2017	Sofosbuvir + Velpatasvir + Voxilaprevir	Genotype 1 to 6	Vosevi®

FDA: Food and Drug Administration; RBV: Ribavirin; IFN: Interferon.

REGRESSION OF FIBROSIS AFTER DAA THERAPY

Liver biopsy is the gold standard to estimate liver fibrosis regression after DAA therapy. In a study by Cheng *et al*[22], the Metavir fibrosis score decreased from F3-F4 to F0-2 in more than 50% of the patients from baseline to post-therapy. Since liver biopsy is an invasive procedure that can be associated with potential adverse events, non-invasive modalities have been developed to monitor hepatic fibrosis[23,24].

Fibrosis markers: Fibrosis-4 and aminotransferase to platelet ratio index

Aminotransferase to platelet ratio index (APRI) and fibrosis-4 (FIB-4) are non-invasive serum fibrosis markers. FIB-4 and APRI values have been shown to decrease significantly during the first four weeks of DAA therapy[22]. The initial reduction in fibrosis may be related to a decrease in hepatic inflammation. They reported that aspartate aminotransferase (AST) and ALT values significantly decreased by 50.8% and 64.1% respectively after 4 wk of DAA therapy and ultimately reaching normal values[22].

Fibroscan or vibration-controlled transient elastography

Vibration-controlled transient elastography is a non-invasive and accurate measuring tool of liver fibrosis. Liver stiffness scores significantly decreased in patients who responded to DAA. Several studies have shown long-term regression of fibrosis over a follow-up period of 2 years.

Rout *et al*[25] reported that high baseline liver stiffness measurements (LSM), low platelet count, and low body mass index (BMI) were independently associated with improvement of LSM values one year after successful therapy. Furthermore, the levels of serum transaminases were not significantly associated with a reduction of LSM on multivariate analysis.

Chan *et al*[26] monitored a cohort of patients for at least a year after completion of DAA therapy to exclude the confounding effect of liver inflammation on LSM. They observed the median intra-patient LSM reduction was 0.5 kPa between the end of therapy and 12 mo after treatment.

Stasi *et al*[27] observed the greatest reduction in stiffness values at end of DAA therapy. The reduction in fibrosis was more gradual thereafter. In this group of patients, the liver stiffness values reduced progressively at 1 year, 2 years after treatment, respectively. Their findings suggested a continued reduction of fibrosis beyond the initial resolution of inflammation.

Several studies reported that patients with advanced fibrosis had significant fibrosis regression after achieving SVR. The reduction was approximately 3.1 kPa in 6-12 mo after achieving HCV cure, and the median decline in liver stiffness was 28.2% (interquartile range of 21.8% to 34.8%)[28]. Despite a reduction from baseline LSM, more than half of the patients remained cirrhotic at week 24 after treatment completion[29]. This result is consistent with previous observations that advanced fibrosis often persists after SVR[30,31].

RISKS OF HCC AFTER HCV CURE

Lack of fibrosis regression

It is crucial to explore the relationship between the lack of fibrosis regression and HCC risk especially in patients with advanced fibrosis and cirrhosis[32,33]. In a study by Ravaioli *et al*[34], 139 patients with HCV-related cirrhosis who achieved SVR after DAA treatment were included to evaluate their HCC risk by comparing LSM at baseline to end of treatment. The majority of the patients were male (65.5%) and genotype 1b (58.3%). Those who developed HCC had in average an 18% reduction in LSM compared to 28.9% among those without HCC (P = 0.005). At multivariate analysis, a less than 30% reduction in LSM was an independent HCC risk factor.

In another study, Kawagishi *et al*[35] evaluated fibrosis regression by LSM in 110 HCV patients who achieved SVR. Regression of liver fibrosis was defined as: A decrease by > 1 stage after DAA therapy in patients with liver fibrosis stage F2 to F4; and no deterioration of fibrosis in patients with liver fibrosis F0/1. They found the rate of regression was lower at 96 wk after SVR among those with higher baseline fibrosis stages.

Hepatic steatosis and non-alcoholic fatty liver disease

Hepatic steatosis is one of the histopathologic features of CHC[36]. Both *in vitro* and *in vivo* studies have shown that HCV core protein expression either in cell cultures or in transgenic mice led to the development of hepatic steatosis, contributing to carcinogenesis[37-39]. Cholet *et al*[40] in their study demonstrated a significant relationship between steatosis and hepatic fibrosis in CHC highlighting the important role played by steatosis in liver disease progression in CHC. This relationship remained significant in multivariate analysis as well[40].

Hepatic steatosis is among the factors associated with increased risk of developing HCC in HCV patients after DAA therapy[41,42]. In a large retrospective study conducted by Peleg *et al*[43] on 515 CHC patients treated with interferon-free DAA regimens, baseline liver steatosis (LS) was significantly associated with all-cause mortality and the development of HCC after treatment. Patients with LS had higher incidence rates of HCC (5.23 cases per 100 person-years, 95%CI: 4.85-5.71) compared to patients with advanced fibrosis (3.51 cases per 100 persons-years, 95%CI: 3.33-3.67). Moreover, patients with LS without advanced fibrosis had higher rates of mortality and HCC compared to those with advanced fibrosis but without steatosis[42]. Kono *et al*[44] concluded in their study of 286 CHC patients that fatty liver along with advanced liver fibrosis is associated with sustained liver damage with abnormal alpha-feto protein (AFP) and ALT levels even after HCV cure. In a prospective study conducted by Noureddin *et al*[45], 47.5% of the HCV patients with SVR had evidence of LS. Long-term follow-up of these patients is critically important to monitor progressive liver disease.

Diabetes mellitus

Diabetes mellitus (DM) is identified as a significant risk factor for HCC in HCV patients after SVR but the mechanism remains unclear[46-48]. There is some evidence suggesting hyperinsulinemia and insulin-dependent signaling pathways are linked to the pathogenesis and progression of HCC. Insulin resistance increases the rate of fibrosis progression in HCV infected patients. Hyperinsulinemia and insulin resistance as a result of cirrhosis can further promote the development of HCC[49]. HCC risk after interferon-induced SVR in patients with DM and cirrhosis had been reported. Subsequently, this association was also noted after DAA therapy. A 3-year follow-up study including 565 CHC patients with cirrhosis treated with DAAs identified diabetes as an independent predictor of *de novo* HCC[50-54]. Degasperi *et al*[47] identified diabetes as a strong independent predictor for *de novo* HCC development and also HCC recurrence in a cohort of 546 HCV patients treated with DAA. On the multivariate analysis, diabetes [hazard ratio (HR): 2.52, 95%CI: 1.08-5.87, P = 0.03] predicted *de novo* HCC as well as HCC recurrence (HR: 4.12, 95%CI: 1.55-10.93, P = 0.004)[47]. Similarly, in another study, Lu *et al*[48] also found that DM had a significant effect on the risk of HCC [adjusted HR (aHR): 1.65, 95%CI: 1.09-2.49]. In contrast, DM was not associated with an increased risk of developing HCC after DAA-induced SVR in studies by Kanwal *et al*[41].

Alcohol

Alcohol is an important HCC risk factor regardless of the presence of HCV. The annual incidence of HCC is higher among patients with alcohol use compared to those

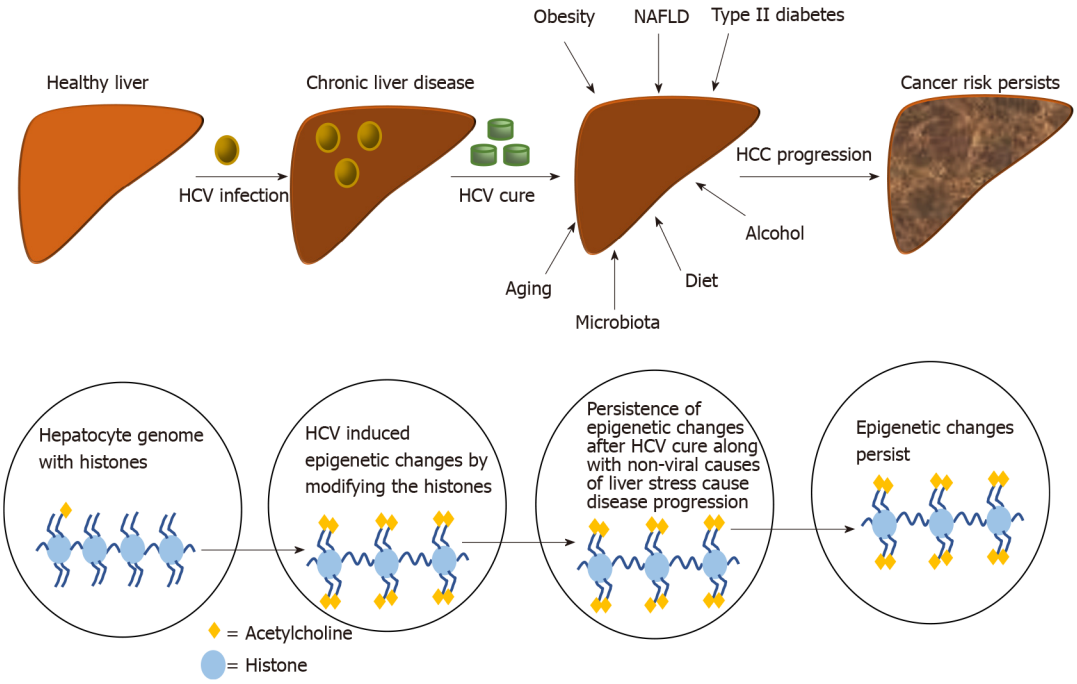


Figure 2 Pathogenesis of hepatocellular carcinoma. HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

without (aHR: 4.73, 95%CI: 3.34-6.68)[41]. Alcohol-induced oxidative stress and the hepatic metabolism of ethanol could increase the conversion of pro-carcinogens to active carcinogens that results in HCC[42]. Caldwell *et al*[55] found that history of heavy alcohol consumption, defined as consumption of more than 2 drinks per day or 14 drinks per week for female; 3 drinks per day or 21 drinks per week for male, had a direct impact on FIB-4 score. It was significantly higher in the group with heavy alcohol abuse compared to no alcohol abuse. A daily intake of ≥ 80 g of ethanol for > 10 years is thought to increase the risk of HCC by approximately five-fold and women are more susceptible to alcohol toxicity than men[56,57]. Alcohol acts synergistically with HCV in accelerating the progression to cirrhosis and liver-related complications [58]. The ethanol's effects on hepatic fibrogenesis persist after HCV cure for those who continue to consume alcohol. A study by Kanwal *et al*[41] reported a higher annual incidence of HCC among patients with alcohol use (1.01%, 95%CI: 0.83-1.19) compared to those without (0.72%, 95%CI: 0.54%-0.91%; aHR: 1.56, 95%CI: 1.11-2.18) after achieving SVR post DAA therapy[41] (Figure 2).

PATHOGENESIS OF HCC AFTER HCV CURE

A number of key pathways are involved in the development of HCV-related HCC: (1) Fibrosis due to continuous necrosis; (2) Immune-surveillance failures attributable to persistent viral replication with immune system escape mechanisms; and (3) Direct carcinogenic effect of HCV proteins which deregulate host cell cycle checkpoints leading to DNA mutations in liver cells[59]. The pathogenesis of HCC after HCV cure remains elusive. A 186-gene expression signature in liver tissue of CHC patients with HCC suggested virus-induced transcriptional reprogramming in the liver leading to carcinogenesis[60,61]. Epigenetic modifications of histones, for example, can lead to chromatin opening and compacting which, in turn, affect gene regulation[62]. Hamdane *et al*[63] investigated HCV-induced epigenetic alterations that might increase HCC risk after DAA treatment in patients and mice with humanized livers. They found that chronic HCV infection induced specific genome-wide changes in H3K27ac. The 5318 modified genes associated with CHC correlated with changes in the expression of mRNAs and proteins. A number of the altered pathways resulting from epigenetic changes persisted after HCV cure with DAAs. Namely, molecular pathways involving tumor necrosis factor α signaling, inflammatory response, G2M checkpoint, epithelial-mesenchymal transition, phosphoinositide 3-kinase, Akt, and mammalian target of rapamycin[63]. This analysis showed that H3K27ac changes observed in HCV-infected patients were partly reversed after cure for those with stage

F2-3 fibrosis. This group shared only 42.5% of the HCV-modified genes. In contrast, in DAA-cured patients with cirrhosis (stage F4), 96.6% of the HCV-induced H3K27ac changes persisted[63]. By performing chromatin immunoprecipitation followed by next-generation sequencing of histone post-translational modifications that are epigenetic markers for active and repressed chromatin, Perez *et al*[64] also demonstrated that HCV infection induces genome-wide epigenetic changes. The "epigenetic signature" persisted after achieving DAA- related cure. Santangelo *et al*[65] examined the impact of DAAs on the ability of exosomal microRNAs (miRs) to modulate the innate immune response in patients with CHC. miR-122 was selectively studied as it is involved in HCV replication and its loss has been associated with HCC development. The study showed that miR-122-5p, miR-222-3p, miR146-5p, miR-150-5p, miR-30C-5p, miR-378a-3p, miR-20a5p were enriched in exosomes derived from the HCV-infected cells. The liver-specific miR-122 levels and the expression of the aforementioned miRs significantly decreased after DAAs therapy[65]. Human HCC cells express vascular endothelial growth factor (VEGF) that functions as a cytokine and affects cancer cell growth and survival[66]. The VEGF expression correlates with liver cancer angiogenesis and proliferative activity. Villani *et al*[66] studied the effect of DAA treatment-induced VEGF on HCC angiogenesis. In this study on 117 cirrhotic patients treated with DAA, a 4-fold increase in VEGF was observed compared to baseline. This significant increase in VEGF could potentially lead to an acceleration of cancer cell proliferation prior to HCV cure and the carcinogenesis remained after DAA even though the VEGF decreased to normal levels 12 wk after DAA treatment (Figure 2).

IDENTIFYING PATIENTS WITH HCC RISK AFTER HCV CURE

Although achieving SVR is the goal of HCV treatment, the risk of developing HCC remains high particularly in patients with advanced fibrosis and cirrhosis[67]. This risk ranges between 1.8% and 2.5% annually. The current guidelines suggest that these patients should undergo HCC surveillance every six months by ultrasound with or without alfa-fetoprotein indefinitely. On the contrary, patients with no or moderate fibrosis who achieved SVR and have no risk behavior could be discharged from specialty care[68]. Methods to identify patients with differential HCC risks can be challenging.

APRI and FIB-4 have been used to assess the HCC risks. These scores, however, were not developed specifically for HCC indication; thus, their accuracy is limited. Transient elastography, similarly, was not designed to detect HCC[68]. Specific score systems designed to predict HCC after HCV cure remain an unmet need.

A group in Japan developed a simple score to identify HCV patients at risk of HCC after achieving SVR[69]. The majority were HCV serotype 1 or 2 patients. They use multivariate analysis to identify predictive variables. They found that age (cutoff 75 years) and post-treatment AFP (cutoff 6 ng/mL) values were independent factors for HCC. Thus, they used a score with 0 and 1 point for each factor: < 75 and > 75 years were set as 0 and 1 point; < 6 and > 6 ng/mL were set as 0 and 1 points respectively. The sum of each factor was considered as the final score. HCC incidence increased significantly with higher scores. In the 0-point group, the incidence of HCC was 0% at 6 mo; 0.3% at 12, 18 and 24 mo; and only 1.26% at 36 mo. In contrast, the risk increased in the 2-point group: 2.88% at 6 mo; 4.92% at 12 mo; 11.61% at 18 mo; and up to 18.37% after 24 mo. This scoring system is simple to apply but needs to be validated prospectively in different patient populations.

In Egypt, Shiha *et al*[70] conducted a prospective study to develop an HCC risk model after SVR. Their model used clinical variables to create scores for low, intermediate and high HCC risk. Each variable was given a score according to its HR. This General Evaluation Score included age (< 54 = 0; > 54 = 1), gender (male = 3.5; female = 0), fibrosis stage (F3 = 1.5; F4 = 3), albumin (> 3.8 g/dL = 0; < 3.8 g/dL = 2) and alpha-fetoprotein levels (< 20 ng/mL = 0; > 20 ng/mL = 3). The score range was between 0 and 12.5. The low-risk group (score < 6) had a 1-year HCC incidence of 0.1%, 1.2% at 2 years and 1.9% at 3 years. The intermediate-risk group (score 6-7.5) had a 1-year incidence of 0.7%, 3.3% at 2 years and 5.8% at 3 years. Finally, the high-risk group (score > 7.5) had a 1-year HCC incidence of 1.2% which increased to 7.1% at 2 years and 9.5% at 3 years. The advantage of this tool is that it uses commonly available clinical variables that can be applied in different settings including low and medium-income populations. This study included only patients with HCV genotype 4. If it is validated in other HCV genotypes and populations, it can be a cost-effective tool for HCC surveillance.

Ioannou *et al*[71] developed different sets of models according to treatment modalities for CHC. For those with DAA-induced HCV cure, the regression model showed that age > 60, platelet count < 61 × 10^4, serum AST/ALT ratio > 8.8 in non-cirrhotic and > 11.01 in cirrhotic; and albumin < 2.9 were major predictive variables for the development of HCC. By applying these variables in the models, the cirrhotic/non-SVR group was predicted to have a 13.1% HCC risk at 2.6-year follow-up; the cirrhotic/SVR group had a 4.5% incidence at 2-year follow-up; the non-cirrhotic/non-SVR had a 4.2% incidence at 3.7-year follow-up; whereas the non-cirrhotic/SVR group had only a low 0.7% HCC risk at 2.3-year follow-up. Given the differential risks according to the clinical characteristics, the HCC screening guidelines could potentially be narrowed to specific risk groups. Although this model was internally validated and is easily available as a web-calculator tool, external validation would be necessary since it was performed using the Veterans Affairs healthcare data only and the majority of patients had HCV genotype 1.

Recently, a model using transient elastography was developed in Spain by Alonso L ópez *et al*[72], they built two dynamic models for patients with advanced fibrosis and cirrhosis who achieved SVR. Their objective was to identify very low HCC risk patients who may not require continued HCC surveillance despite the presence of advanced fibrosis prior to therapy. The first model included baseline albumin, baseline and 1-year follow-up elastography. Given that elastography may not be available in every setting, the second model included serological markers only: Baseline albumin, baseline and 1-year follow-up FIB-4 and 1-year gamma-glutamyl transferase. They found that both models were useful as predictors of HCC. Moreover, after stratification of risk assigned by scoring each variable in both models, the ones who scored 0 had 0%-0.4% risk of developing HCC. The ability to accurately identify those at very low HCC risk could effectively stratify patients for HCC surveillance.

Alpha-fetoprotein is the most available HCC biomarker. Its sensitivity and specificity are very variable[73]. Recent studies have shown sphingolipids as potential biomarkers to detect hepatic decompensation in cirrhotic patients[74]. Two types of sphingolipids - C16-ceramide and sphingosine-1-phosphate - have been applied as HCC biomarkers in cirrhotic patients. Mücke *et al*[75] in Germany evaluated sphingolipids as early predictive HCC biomarkers in HCV patients with cirrhosis who had achieved SVR. They identified C16Cer as an independent biomarker for early detection of *de novo* HCC in both AFP-positive or AFP-negative patients. Although this finding seems novel and promising, prospective studies are needed to clarify the association between sphingolipids and carcinogenesis.

In the area of deep learning, Ioannou *et al*[76] utilized recurrent neural network (RNN) models to identify patients at high risk of developing HCC for at least a 3-year follow-up period after HCV cure. They used two types of variables: Baseline and longitudinal ones to evaluate the risk progression. They compared three models: Cross-sectional logistic regression (LR), longitudinal LR and RNN. The area under the receiver operating characteristic curve for these groups was 0.67, 0.70 and 0.80 respectively. The RNN model was superior to the conventional LR models and could be a promising tool after computational refi-nement.

CONCLUSION

In the DAA era, the development of HCC remains a significant concern especially among those with advanced hepatic fibrosis. A number of factors including diabetes mellitus, underlying non-alcoholic fatty liver disease and alcohol consumption have been associated with progression to HCC after HCV cure. Promising HCC predictive models are being developed but most require validation and standardization. The pathogenesis of HCC after HCV cure remains poorly understood. The understanding of the molecular mechanisms leading to HCC could facilitate the identification of novel biomarkers for early HCC detection.

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MINIREVIEWS

Artificial intelligence in the diagnosis and management of colorectal cancer liver metastases

Gianluca Rompianesi, Francesca Pegoraro, Carlo DL Ceresa, Roberto Montalti, Roberto Ivan Troisi

ORCID number: Gianluca Rompianesi 0000-0003-0756-8013; Francesca Pegoraro 0000-0003-2162-7315; Carlo DL Ceresa 0000-0002-8702-3744; Roberto Montalti 0000-0002-3915-3851; Roberto Ivan Troisi 0000-0001-6280-810X.

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Gianluca Rompianesi, Francesca Pegoraro, Roberto Ivan Troisi, Division of Hepato-Bilio-Pancreatic, Minimally Invasive and Robotic Surgery, Department of Clinical Medicine and Surgery, Federico II University Hospital, Naples 80125, Italy

Carlo DL Ceresa, Department of Hepato-Pancreato-Biliary Surgery, Oxford University Hospitals NHS Foundation Trust, Oxford OX3 9ES, United Kingdom

Roberto Montalti, Division of Hepato-Bilio-Pancreatic, Minimally Invasive and Robotic Surgery, Department of Public Health, Federico II University Hospital, Naples 80125, Italy

Corresponding author: Gianluca Rompianesi, FEBS, MD, PhD, Assistant Professor, Surgeon, Division of Hepato-Bilio-Pancreatic, Minimally Invasive and Robotic Surgery, Department of Clinical Medicine and Surgery, Federico II University Hospital, *via* Pansini 5, Naples 80125, Italy. gianlucarompianesi@gmail.com

Abstract

Colorectal cancer (CRC) is the third most common malignancy worldwide, with approximately 50% of patients developing colorectal cancer liver metastasis (CRLM) during the follow-up period. Management of CRLM is best achieved *via* a multidisciplinary approach and the diagnostic and therapeutic decision-making process is complex. In order to optimize patients' survival and quality of life, there are several unsolved challenges which must be overcome. These primarily include a timely diagnosis and the identification of reliable prognostic factors. Furthermore, to allow optimal treatment options, a precision-medicine, personalized approach is required. The widespread digitalization of healthcare generates a vast amount of data and together with accessible high-performance computing, artificial intelligence (AI) technologies can be applied. By increasing diagnostic accuracy, reducing timings and costs, the application of AI could help mitigate the current shortcomings in CRLM management. In this review we explore the available evidence of the possible role of AI in all phases of the CRLM natural history. Radiomics analysis and convolutional neural networks (CNN) which combine computed tomography (CT) images with clinical data have been developed to predict CRLM development in CRC patients. AI models have also proven themselves to perform similarly or better than expert radiologists in detecting CRLM on CT and magnetic resonance scans or identifying them from the noninvasive analysis of patients' exhaled air. The application of AI and machine learning (ML) in diagnosing CRLM has also been extended to histopathological examination in order to rapidly and accurately identify CRLM tissue and

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its different histopathological growth patterns. ML and CNN have shown good accuracy in predicting response to chemotherapy, early local tumor progression after ablation treatment, and patient survival after surgical treatment or chemotherapy. Despite the initial enthusiasm and the accumulating evidence, AI technologies' role in healthcare and CRLM management is not yet fully established. Its limitations mainly concern safety and the lack of regulation and ethical considerations. AI is unlikely to fully replace any human role but could be actively integrated to facilitate physicians in their everyday practice. Moving towards a personalized and evidence-based patient approach and management, further larger, prospective and rigorous studies evaluating AI technologies in patients at risk or affected by CRLM are needed.

Key Words: Colorectal cancer; Liver metastases; Artificial intelligence; Machine learning; Deep learning; Neural networks; Radiomics

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Core tip: The digitalization of healthcare generating huge amount of data set the ground for the progressive ubiquitous application of artificial intelligence (AI) technologies in healthcare. AI analyses can assist clinicians in all phases of colorectal liver metastases natural history: From predicting their occurrence, to increasing diagnostic accuracy or estimating recurrence risk after treatment and patient outcome. The implementation of AI resources supports the contemporary paradigm shift that sees healthcare focus moving from a generalized, disease-oriented to an individual, patient-centered, precision medicine approach.

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INTRODUCTION

Colorectal cancer liver metastases

Colorectal cancer (CRC) is the most common gastrointestinal cancer, the third most frequently diagnosed malignancy (10.0%) overall, and the second highest cause of cancer-related deaths (9.4%), with incidences varying significantly worldwide[1,2]. CRC development is predominantly sporadic, with patient age, environmental and genetic factors associated with a significantly increased risk[3,4]. Over 20% of newly diagnosed CRC patients have distant metastases at presentation[5], with estimated 5-year survival dropping from 80%-90% in patients with local disease to a dismal 10%-15% in those with metastatic spread[6]. The liver is the preferential metastatic site, due to its anatomical proximity and the portal systemic circulation. This results in 25%-50% of CRC patients developing liver metastasis during the course of the disease[7,8]. In cases of synchronous resectable colorectal cancer liver metastasis (CRLM), the treatment options range from the traditional staged approach, where the primary tumor is resected prior to systemic chemotherapy and liver metastasis resection, to the combined approach of bowel and liver resection during the same procedure, or the "liver first" approach[9]. Irrespective of the timing of the surgical resection, surgery in combination with chemotherapy is the optimal treatment for CRLM, but only 25% of patients are suitable candidates for resection at diagnosis[8,10]. In patients not amenable to surgery, chemotherapy is the usual treatment of choice, with the potential to render 10%-30% of tumors technically resectable through a good response and downsizing[11]. CRLM management is multidisciplinary, with oncologists, surgeons, radiologists and pathologists playing pivotal roles in the complex diagnostic and therapeutic decision-making processes aimed to achieve the best possible outcome for the patient[12]. In such a complex oncological scenario, with unsolved challenges in

timely diagnosis, reliable prognostic factor identification and optimal treatment selection, there is a strong need for a precision-medicine, personalized approach in order to optimize patients' survival and quality of life. The recent progressive implementation of artificial intelligence (AI) in healthcare has been welcomed with enthusiasm by both healthcare professionals and the general public; however, there remain several issues which are yet to be solved. AI has the potential to overcome some of the current practice limitations, and to play a crucial role in all steps of the management of CRLM but its clinical benefits have yet to be clearly established and validated.

The aim of this review is to summarize and analyze the available evidence on the application of AI technologies in the diagnosis and management of patients affected by CRLM.

AI

The term AI encompasses all the possible applications of technologies in simulating and replicating human intelligence[13]. These endless applications range from everyday life to finance and economics[14] or various medical fields, thanks to the advances in computational power and the collection and storage of large amounts of data in healthcare. After being adequately programmed and trained, AI has the potential to outperform clinicians in some tasks in terms of accuracy, speed of execution and reduced biases[15]. AI has therefore progressively demonstrated its potential across all human lifespan; from the optimization of embryo selection during *in vitro* fertilization[16] to the prediction of all-cause mortality[17]. The revolutionary potential of these technologies in healthcare has generated great interest in researchers, professionals and industries, with currently over 450 AI-based medical devices approved in Europe or the United States[18]. Nevertheless, the surge of AI and its implemen-tation in clinical practice has been accompanied by several issues including legal considerations regarding security and data, software transparency, flawed algorithms and inherent bias in the input data[13,19].

Machine learning

The replication of human intelligence by AI with the utilization of data-driven algorithms that have been instructed and self-train through experience and data analysis is generally defined as machine learning (ML)[13]. After been programmed, ML can find recurrent patterns in large amount of appropriately engineered data and progressively learn and independently improve performance accuracy without human intervention. The ML algorithms are generally classified in supervised learning (the most frequent one, which utilizes classified data), unsupervised learning (where algorithms can independently identify patterns in data without previous classification), semi-supervised learning (can use a combination of both labelled and unlabeled data) and reinforcement learning (uses estimated errors as proportional rewards or penalties to teach algorithms). Deep learning (DL) is a class of ML techniques that has the ability to directly process raw data and perform detection or classification tasks automatically without the need for human intervention. The sets of algorithms utilized by DL are generally artificial neural networks (ANNs) constituted by several layers that elaborate inputs with weights, biases (or thresholds) and deliver an output. ML models can be combined with the large amount of qualitative and quantitative information mined from medical images (radiomics) and clinical data to assist clinicians in evidence-based decision making processes[20].

PREDICTIVE AI MODELS FOR THE DEVELOPMENT OF CRLM

A significant proportion of patients affected by CRC will develop CRLM during the follow-up period[21], but only about a quarter of them will be eligible for surgical resection and therefore potential cure[22]. Being able to identify the subgroup of patients at higher risk of CRLM development could allow the adoption of individualized and more intense screening protocols and adjuvant therapies.

The Radiomics Intelligent Analysis Toolkit-based analysis platform built by Li *et al* [23] allowed the construction of individualized nomograms able to combine maximum-level enhanced computed tomography (CT) images in the portal venous phase and patients' clinical information [age, sex, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9] to predict the development of CRLM in patients with CRC. The area under the receiver operating characteristic (AUROC) score obtained from the analysis of 100 patients (50 with CRLM and 50 controls) was 0.899 [95% confidence

interval (CI): 0.761-1.000] on the test set in a total execution time of 270 s. The ML predictive models built by Taghavi[24] including radiomics and a com-bination of radiomics with clinical features (contrast-enhanced portal venous phase CT of the liver or abdomen with age, sex, primary tumor site, tumor stage, nodal stage, CEA at primary diagnosis, administration of adjuvant/neoadjuvant chemotherapy) of 91 patients (24 of which developed metachronous CRLM), both presented an area under the curve (AUC) in the validation cohort of 86% (95%CI: 85%-87%) in predicting the development of CRLM within 24 mo. The convolutional neural network (CNN) model developed by Lee *et al*[25] in their retrospective, cross-sectional study in 2019 patients who underwent curative colectomy for stage I-III CRC was able to predict 5-year metachronous liver metastasis occurrence with a mean AUC of 0.747 when combining the analysis of the abdominal CT scan taken before the colectomy for clinical staging and clinical features (age, sex, tumor stage, nodal stage).

AI MODELS FOR THE DIAGNOSIS OF CRLM

Prompt diagnosis of CRLM at an early stage gives patients the best chances of effective treatment and a superior outcome. One of the key steps in the diagnostic process is tumor segmentation, with nodule volume being a better predictor than diameter[26]. This process is usually done manually but requires a significant expertise, is operator-dependent and time-consuming. In this setting, semiautomatic tumor segmentation methods based on texture analysis have been developed[26] in order to take full advantage of AI's unique potential to increase sensitivity and specificity of metastatic tumor detection[27].

CT radiomics models

Starting with a manual tumor/nontumor class prediction voxel classification, a deformable surface model fitting the tumor boundaries is instigated[27]. A multilayer perceptron feed-forward neural network model concurrently learns per-voxel image features and classifications and, after being trained, it performs a semiautomatic per-tumor segmentation on CT scans. The accuracy of the model resulted in 0.88 ± 0.11, with a sensitivity of 0.84 ± 0.13 and a specificity of 0.92 ± 0.16. The same group in 2019 published the results of a retrospective analysis of a fully CNN for liver lesion detection and segmentation on CT scans with a sensitivity of 71% and 85% and a positive predictive value of 83% and 94% for lesions bigger than 10 mm and 20 mm in diameter, respectively[28]. CRLM is most commonly diagnosed in the venous phase of contrast-enhanced CT scan, as it appears hypodense, with or without peripheral rim enhancement and calcification. Portal-venous phase scans are most reliable in the detection of CRLM, with a sensitivity of approximately 85% for helical CT[29], and such diagnostic power lies in an optimal timing of image acquisition after a delay following contrast intravenous injection. Different equipment, protocols, patient's body habitus and cardiovascular system function result in high variability and impact on measurement accuracy in the absence of reliable automatic timing quantification. Ma *et al*[30] designed a fully automatic DL CNN that in a 3-s timespan can recognize the optimal portal venous phase acquisitions on CT scans with an AUC of 0.837 (95%CI: 0.765-0.890) in the validation set and an AUC of 0.844 (95%CI: 0.786-0.889) in the external validation set. This is aimed to improve image quality, which is crucial for the detection and characterization of liver lesions and the evaluation of parameters identified as predictors of treatment response and outcome, such as the tumor size, enhancement and vascularity[30]. The DL-based algorithm of Kim *et al*[31] aimed at detecting CRLM without human manipulation and fed by raw data from CT images, showed a sensitivity of 81.82%, comparable to that of radiologists (80.81%, $P = 0.80$), but with significantly more false positives per patient (1.330 *vs* 0.357, $P < 0.001$).

A challenging scenario that can occur in 16%-26% of patients with CRC is when the staging CT scan shows small hypoattenuating hepatic nodules defined as too small to characterize. Further imaging such as magnetic resonance (MR), repeat CT after a time interval, or performing a biopsy can delay treatment, increase costs, remain inconclusive, or have the risk of complications and tumor seeding. However, obtaining a diagnosis is of paramount importance given that 9%-14% of these nodules will prove to be malignant[32,33]. CNN could represent a useful adjunct in the characterization of small hypoattenuating liver lesions, and the model developed by Khalili *et al*[34] presents an AUROC similar to the one of expert radiologists, with better diagnostic confidence (significantly lower proportion of nodules rated in the low confidence zone, 19.6 *vs* 38.4%).

MR radiomics models

Despite CT imaging being the most widely used modality in detecting metastatic liver tumors, it can still miss up to 25% of CRLM[35] and MR has progressively gained an established role thanks to the high sensitivity and specificity and absence of ionizing radiations[36,37]. AI utilizing CNN for liver segmentation and CRLM detection could assist radiologists in this complex task and potentially reduce the manual liver lesion detection failure rate of 5%-13%[38]. The CRLM detection method developed by Jansen *et al*[38] is based on a fully CNN with an automatic liver segmentation and the analysis of both dynamic contrast-enhanced and diffusion-weighted MR images in 121 patients. It resulted in an impressive a high sensitivity of 99.8% and a low number of false positives.

Volatile-organic-compound-based models

Interestingly, a ML model has been used by Steenhuis *et al*[39] to analyze data from a retrospective cohort of 62 patients following curative CRC resection to detect CRLM development or local recurrence. The volatile organic compounds (VOCs) from patients' exhaled air are gaseous products of metabolism known to be altered by pathological processes, such as abnormal cell growth, necrosis or intestinal microbioma alteration, and have been evaluated by ML techniques for pattern recognition. This pilot study, despite the limitations due to the small sample size and lack of histological confirmation in about a quarter of patients, showed that the noninvasive, repeatable, and easily applicable eNose analysis was able to identify CRLM or local recurrence with a sensitivity of 0.88 (95%CI: 0.69-0.97), specificity of 0.75 (95%CI: 0.57-0.87), and an overall accuracy of 0.81. Miller-Atkins *et al*[40] combined VOC analysis and demographic data (age and sex) in a predictive model developed using random forest ML and cross-validation that was able to identify patients with CRLM from healthy controls with a classification accuracy of 0.86, specificity of 0.94 but a sensitivity limited to 0.51.

Histology-based models

The applications of AI and ML in diagnosing CRLM have been extended to histopathological examination in order to rapidly and accurately identify CRLM tissue. A probe electrospray ionization-mass spectrometry and ML model was able to distinguish CRLM (103 samples) from noncancer liver parenchyma (80 control samples) with an accuracy rate of 99.5% and a AUROC of 0.9999[41]. CRLM patients are a heterogeneous group with considerable variations, including histopathological growth patterns (HGPs) and corresponding microvasculature[42]. The two predominant types of HGPs are the desmoplastic and replacement, with the pushing and mixed types being far less common. Once accurately determined by analyzing the interface between the tumor cells and the nearby normal liver, HGPs can represent a useful prognostic and predictive biomarker for response to therapy and overall survival[43-46]. The MR-based radiomics model developed by Han *et al*[47] aims at preoperatively identifying HGP of CRLM with an AUC of 0.906 in the internal validation cohort when the analysis is performed on the tumor-liver interface zone.

AI MODELS FOR TREATED CRLM

Surgical resection offers patients presenting with synchronous or metachronous CRLM the only potential for cure and a superior long-term survival[48] but unfortunately only a fraction of newly diagnosed patients are suitable for surgery. Liver-directed ablative therapies have progressively gained a role in treating nonsurgical candidates with acceptable safety and efficacy profiles[49]. In spite of this, recurrence after CRLM treatment represents a major problem, with an overall risk of local or distant tumor development after surgical resection or ablation as high as 70%-80%, with early recurrences being associated with a poorer prognosis[50,51]. Chemotherapy is of paramount importance in determining outcome of patients with either resectable or unresectable CRLM[8] and can convert up to one third of initially unresectable patients to receive potentially curative treatment[52].

AI models predicting response to chemotherapy

A reliable assessment of response to chemotherapy is of paramount importance for the personalized treatment decision-making process to determine eligibility for surgery, or the need for second-line treatments[53]. Discriminating responsive from unresponsive

nodules or new lesions on the CT scan often represents a challenging task for radiologists, therefore Maaref *et al*[54] developed a fully automated framework based on DL CNN that achieved an accuracy of 0.91 (95%CI: 0.88-0.93) for differentiating treated and untreated lesions, and 0.78 (95%CI: 0.74-0.83) for predicting the response to a FOLFOX + bevacizumab-based chemotherapy regimen. Similarly, the DL ra-diomics model by Wei *et al*[55] was able to predict response to chemotherapy (CAPEOX, mFOLFOX6, FOLFIRI or XELIRI regimens) of CRLM based on contrast-enhanced CT according to the response evaluation criteria in solid tumors with an AUC in the validation cohort of 0.820 (95%CI: 0.681-0.959) that increases to 0.830 (95%CI: 0.688-0.973) combining the DL-based model with the CEA serum level. Human epidermal growth factor receptor 2 amplification or overexpression is found in 2%-6% of stage 2/3 CRC patients and treatment with trastuzumab and lapatinib has proven to be beneficial in the 70% of metastatic cases[56]. Giannini *et al*[57] published the results of an ML algorithm predicting the therapeutic response in such a subgroup of patients with an overall sensitivity of 92% (95%CI: 75%-99%) and specificity of 86% (95%CI: 42%-100%). The radiomics-based prediction model for the response of CRLM to oxaliplatin-based chemotherapy developed by Nakanishi *et al*[58] with radiomics features extracted from the pre-treatment CT scans, significantly discriminated good responders (AUC: 0.7792, 95%CI: 0.618-0.941).

AI models predicting recurrence after local ablative therapies

In order to predict early local tumor progression after ablation treatment of up to five nodules per patient with a maximum diameter of 30 mm, Taghavi *et al*[59] developed a ML-based radiomics analysis of the pretreatment CT scan combined with patients' clinical features that showed a concordance index in the validation cohort of 0.79 (95%CI: 0.78-0.80).

AI MODELS PREDICTING SURVIVAL IN CRLM PATIENTS

AI models predicting overall survival

The systematic comparative analysis of quantitative imaging biomarkers based on the geometric and radiomics analysis of the liver tumor burden by Mühlberg *et al*[60], performed on a retrospective cohort of 103 patients with CRLM with automated segmentation of baseline contrast-enhanced CT images, showed that the tumor burden score (TBS) had the best discriminative performance for 1-year survival (AUC: 0.70; 95%CI: 0.56-0.90). The TBS[61] is calculated combining tumor number and maximum diameter through the Pythagorean theorem [TBS^2 = (maximum tumor diameter)^2 + (number of liver lesions)^2]. An ML method has been used by Hao *et al*[62] to analyze whole-genome methylation data to predict cancer *versus* normal tissue of four common tumors (including 29 of 30 CRLMs) with > 95% accuracy and patient prognosis and survival through DNA methylation analysis.

AI models predicting survival after chemotherapy

Anti-epidermal growth factor receptor (EGFR) therapies are an effective option for RAS wild-type mutational status CRLM, but there is a need for reliable biomarkers that can estimate the balance between risks and clinical benefits of such therapies in individual patients[63]. Dercle *et al*[64] developed an AI model that through ML could create a signature that evaluated a change in tumor phenotype on interval CT scan images (baseline to 8 wk). The resultant model was able to successfully predict both sensitivity to anti-EGFR therapy (0.80; 95%CI: 0.69-0.94) and overall survival ($P < 0.05$).

AI models predicting survival after surgical resection of CRLM

The ANN model constructed by Spelt *et al*[65] retrospectively analyzed a single-center cohort of 241 patients who underwent liver resection for CRLM. Six of the 28 potential risk variables (age, preoperative chemotherapy, size of largest metastasis, hemorrhagic complications, preoperative CEA level and number of metastases) were selected by the ANN model to predict survival more accurately than the Cox regression model, with C-index of 0.72 *versus* 0.66. Paredes *et al*[66] in 2020 published the results of their ML recurrence-free prediction model for patients with CRLM undergoing curative-intent resection using clinical, pathological and morphological tumor characteristics with genetic Kirsten rat sarcoma 2 viral oncogene homolog information. The model, built on the analysis of 1406 multi-institutional patients undergoing liver resection, showed a discriminative ability to predict the recurrence risk at 1, 3 and 5 years (AUROC of

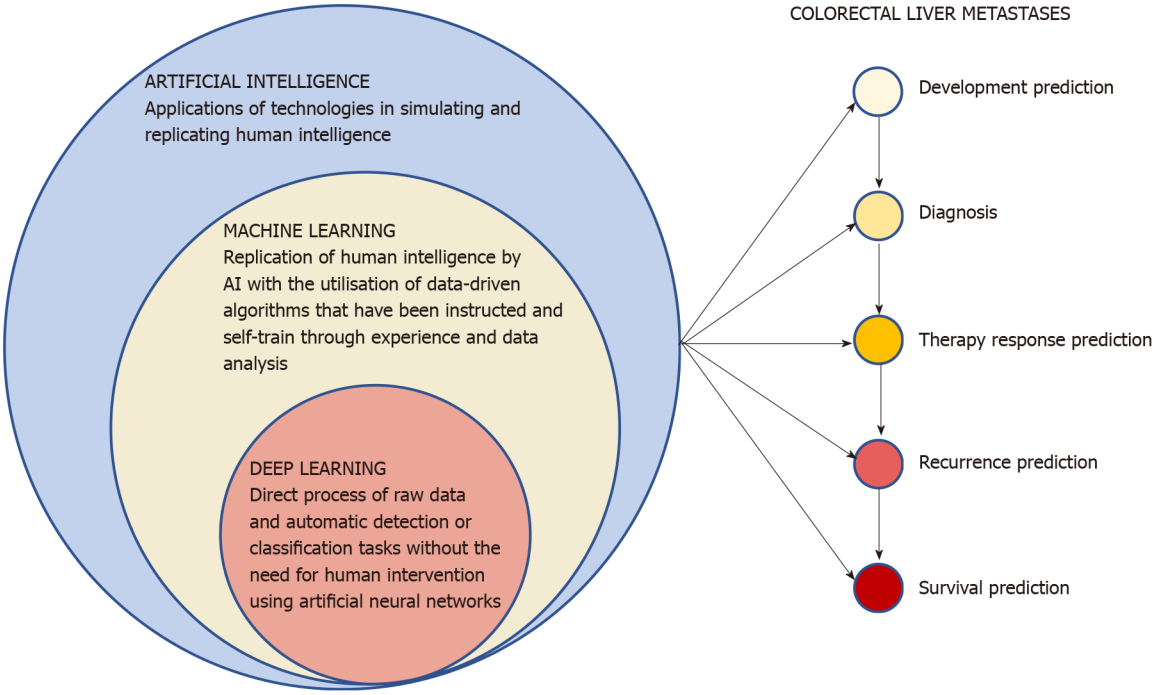


Figure 1 Possible applications of artificial intelligence technologies in the diagnosis and management of colorectal liver metastases. AI: Artificial intelligence.

0.693, 0.669 and 0.669, respectively) more accurate than the ones of Fong[67] and Vauthey[68] scores.

LIMITATIONS

In spite of AI's clear potential there remain several unresolved issues and limitations. These include the potential for artefacts in radiomics analyses to affect the results, the ethical and legal considerations, the definition of minimal accuracy rates and safeguards necessary to ensure public safety. Privacy, sensitive data protection and confidentiality need to remain the unmovable cornerstone of patient rights even in the digitalized era, but at the same time, some limitations on data utilization may affect the necessary linkages to prevent biases or errors in AI-driven analyses. There is a strong need from regulatory bodies for clear guidance during the AI-driven transformation of healthcare in order to take full advantage of the potential major improvements in individual and public health, while ensuring trust, safety and transparency. There is a significant variability in the algorithms investigated so far, as well as heterogeneity in the relatively small sample size of the population on which they have been trained and tested (Table 1). Analyses on large registries or national and international collaborations with data sharing could overcome part of the current limitations that limit the formal recognition of AI as a reliable and reproducible application in clinical scenarios.

CONCLUSION

The progressive widespread availability of high-performance computing, together with the accessibility to a large amount of data constantly generated as the result of the increase in the digitalization, set the ground for the ubiquitous implementation of AI technologies in contemporary healthcare. The fields of medical and surgical oncology have welcomed with enthusiasm the advent of augmented medicine with numerous studies investigating its potential, also given the high complexity and diversity of cancer patients. CRC makes no exception and still represents a leading cause of cancer-related death due to its high incidence, rapid progression potential and biological heterogeneity that advocate the need for reliable and individualized diagnostic, prognostic and treatment selection tools. Recent years have seen AI technologies tested

Table 1 Summary of the studies considered in this review

Author	Study design	AI model type	Data source	Total sample size/training cohort/validation cohort	AUC training/AUC validation	Sensitivity/specificity	PPV/NPV	Accuracy
CRLM development								
Li *et al*[23] (2020)	Retrospective; Single center	Radiomics/ML	CT images ± clinical data	100/NA/80	0.90/0.906	81%/84%	85%/79%	NA
Taghavi *et al*[24] (2021)	Retrospective; Multicenter	Radiomics/ML	CT images ± clinical data	91/70/21	0.95[2]-0.68[3]-0.95[4]/0.86[2]-0.71[3]-0.86[4]	NA/NA	NA/NA	NA
Lee *et al* [25] (2020)	Retrospective; Single center	Radiomics/CNN	CT images ± clinical data	2019/1413/606	NA/0.606[2]-0.709[3]-0.747[4]	NA/NA	NA/NA	NA
Diagnosis								
Vorontsov *et al*[26] (2017)	Retrospective; Single center	Radiomics/CNN	CT images	40/32/8	NA/NA	84%/92%	NA/NA	88%
Vorontsov *et al*[28] (2019)	Retrospective; Single center	Radiomics/CNN	CT images	156/115/15	NA/NA	59%[5]/NA	80%[5]/NA	NA
Ma *et al* [30] (2020)	Retrospective; Multicenter	CNN	CT images	909/479/202 (228[6])	NA/0.837-0.844[6]	82%[6]/74%[5]	75%[6]/81%[6]	NA
Kim *et al* [31] (2021)	Retrospective; Single center	DL	CT images	587/502/85	NA/0.631	81.82%/22.22%	NA/NA	NA
Khalili *et al*[34] (2020)	Retrospective; Single center	CNN	CT images ± liver metastatic status	199/150/49	NA/0.84-0.95[7]	(81.5%-81.5%[7])/(76.2%-96.4%[7])	NA/NA	78.3%; 90.6%[6]
Jansen *et al*[38] (2019)	Retrospective; Single center	CNN	MRI images	121/334[1]/86[1]	NA/NA	99.8%/NA	NA/NA	NA
Steenhuis *et al*[39] (2020)	Retrospective; Single center	ML	VOCs	62/NA/NA	NA/0.86	88%/75%	72%/90%	81%
Miller-Atkins *et al*[40] (2020)	Prospective; Single center	ML	VOCs	296/284/NA	NA/NA	51%/94%	NA/NA	86%
Kiritani *et al*[41] (2021)	Retrospective; Single center	ML	Histologic markers	183/NA/40	NA/0.999	100%/99%	NA/NA	99.5%
Han *et al* [47] (2020)	Retrospective; Single center	Radiomics/ML	MRI images ± clinical data	107/61[1]/31[1]	0.974[2]-0.659[3]-0.971[4]/0.912[2]-0.676[3]-0.909[4]	95.2%[2]-57.1%[3]-95.2%[4]/80.0%[2]-70.0%[3]-70.0%[4]	NA/NA	90.3%[2]; 61.3%[3]; 87.1%[4]
Chemotherapy response								
Maaref *et al*[54] (2020)	Retrospective; Single center	DL CNN	CT images	202/70%/10%	0.97/0.88	98%/54%	NA/NA	91%[8]; 78%[9]
Wei *et al* [55] (2021)	Retrospective; Single center	Radiomics/DL	CT images ± CEA	192/144/48	0.903[10]-0.935[11]/0.820[10]-0.830[11]	90.9%/73.3%	88.2%/78.6%	85.4%
Giannini *et al*[57] (2020)	Retrospective; Multicenter	Radiomics/ML	CT images	38/28/10	NA/NA	92%/86%	96%/75%	NA
Nakanishi *et al*[58] (2021)	Retrospective; Single center	Radiomics	CT images	42/94[1]/32[1]	0.8512/0.7792	NA/NA	NA/NA	NA
Local ablative therapies efficacy								
Taghavi *et*	Retrospective;	Radiomics/ML	CT images	90/63/27	NA/0.78[2]-	NA/NA	NA/NA	NA

al[59] (2021)	Single center				0.56[3]-0.79[4]			
Survival prediction								
Mühlberg *et al*[60] (2021)	Retrospective; Single center	Radiomics/ML	CT images ± WLTB ± TBS	103/NA/NA	NA/0.70[12] –0.73[13]-0.76[14]	NA/NA	NA/NA	NA
Hao *et al* [62] (2017)	Retrospective; Multicenter	ML	DNA methylation	1792[1]/NA/884[1] (718[1,6])	NA/NA	NA/NA	NA/NA	98.4%
Dercle *et al* [64] (2020)	Retrospective; Multicenter	ML	CT images	667/438/229	0.83/0.80	80%/78%	NA/NA	NA
Spelt *et al* [65] (2013)	Retrospective; Single center	ANN	Clinical variables	241/NA/NA	NA/NA	NA/NA	NA/NA	72%
Paredes *et al*[66] (2020)	Retrospective; Multicenter	ML	Clinical variables	1406/703/703	0.527[15]-0.525[16]-0.693[17]/0.524[15] -0.501[16]-0.642[17]	NA/NA	NA/NA	NA

[1]Number of lesions.
[2]Model based on radiomics data only.
[3]Model based on clinical data only.
[4]Model based on both radiomics and clinical data.
[5]Per patient values.
[6]Values calculated on the external validation set.
[7]Model based on both convolutional neural network and liver metastatic status.
[8]For differentiating treated and untreated lesions.
[9]For predicting the response to a FOLFOX + bevacizumab-based chemotherapy regimen.
[10]Model based on both deep learning and radiomics signature.
[11]Model based on deep learning and radiomics signature considering carcinogenic embryonic antigen values.
[12]Model based on tumor burden score.
[13]Model based on geometric metastatic spread of whole liver tumor burden.
[14]Model based on the Aerts radiomics prior model.
[15]Model based on Fong/Blumgart clinical risk score for predicting 1-year recurrence.
[16]Model based on Brudvik–Vauthey clinical risk score for predicting 1-year recurrence.
[17]Model based on Paredes–Pawlik clinical risk score for predicting 1-year recurrence.
AI: Artificial intelligence; ANN: Artificial neural network; AUC: Area under the curve; CEA: Carcinogenic embryonic antigen; CNN: Convolutional neural network; CRLM: Colorectal cancer liver metastases; DL: Deep learning; ML: Machine learning; NPV: Negative predictive value; PPV: Positive predictive value; TBS: Tumor burden score; VOCs: Volatile organic compounds; WLTB: Whole liver tumor burden.

by researchers in all phases of the CRLM natural history, aiming at overcoming the current difficulties and limitations faced by the multidisciplinary team responsible of the patients' care (Figure 1). The possibility of identifying the subgroup of patients at higher risk of CRLM development before the occurrence of the disease from the radiomics baseline CT scan analysis with high accuracy (AUC ≥ 0.75) and in less than 5 min could give such patients the best chances of an early diagnosis, more effective treatment, and therefore, a better outcome thanks to a personalized approach[23-25]. Radiomics has also demonstrated a great potential in assisting the radiologists in diagnosing CRLM from CT and MRI scans also by optimizing the identification of the optimal phases for lesions recognition and characterizing small nodules of uncertain nature[27-31,34,38]. A more efficient diagnostic process would help reduce timings and costs, resulting in a potential benefit for both patients and healthcare systems. AI application in order to rapidly and accurately identify CRLM tissue and its different histopathological growth patterns[41,47] could give a significant contribution towards a rapid oncological individualized approach and treatments. AI technologies have also shown potential as a prognostic and outcome tool, predicting with good accuracy response to chemotherapy[54,55,57,58], early local tumor progression after ablation treatment[59], and patient survival after surgery or chemotherapy[60,64-66].

The possibility of reducing human factors and error, increase accuracy and contain timings and costs while adopting a personalized medicine approach is undoubtedly fascinating and appealing, but despite showing promising results, the role of AI in CRLM patients has not yet been fully elucidated. The implementation of AI resources supports the contemporary paradigm shift that sees healthcare focus moving from a generalized, disease-oriented to an individual, patient-centered, precision medicine approach. The effectiveness of ML models lie on a rigid framework in which a well-defined problem and ground truth along with quantitative objective measures to train and validate the algorithm are needed, making the process efficient but rigid. There is

also a balance to be struck between the accuracy and artificial logic and the risk of AI becoming less intelligible and explainable. On the other hand, AI medical technologies could represent a way to enable patients to take ownership of their own care, increasing participation and autonomy for a more personalized approach.

AI will likely affect the immediate future of medicine and patients' management, but rather than replacing the human roles, it will probably be aimed to assist and facilitate physicians in their practice, while being supervised to ensure maximum safety. This could be in the context of diagnostic uncertainty or to assist in planning optimal treatment strategies. A possible future development would be to improve diagnosis and management through the AI analysis and integration of clinical information, radiomic and genetic data thanks to the recent developments in gene sequencing and liquid biopsies, that have showed great potential in gastrointestinal tumors including CRLM[69-72]. A personalized holistic approach providing reliable data for the diagnosis, management and outcome estimation of cancer patients would assist clinicians in the prevention as well as selecting the most appropriate individualized treatment that would grant the patient the best outcome as well as helping patients to make fully informed decisions.

In order to continue to pursue the ambitious goal of improving patients' care through AI healthcare technologies, further larger, prospective, randomized controlled and rigorous studies are needed.

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ORIGINAL ARTICLE

Basic Study

Focal adhesion kinase-related non-kinase ameliorates liver fibrosis by inhibiting aerobic glycolysis *via* the FAK/Ras/c-myc/ENO1 pathway

Tao Huang, Yuan-Qing-Xiao Li, Ming-Yu Zhou, Rui-Han Hu, Gao-Liang Zou, Jian-Chao Li, Shu Feng, Yong-Mei Liu, Chang-Qin Xin, Xue-Ke Zhao

ORCID number: Tao Huang 0000-0001-6849-5378; Yuan-Qing-Xiao Li 0000-0003-3189-1830; Ming-Yu Zhou 0000-0002-51305950; Rui-Han Hu 0000-0003-0668-2025; Gao-Liang Zou 0000-0002-9460-0802; Jian-Chao Li 0000-0003-2274-3369; Shu Feng 0000-0002-8615-1840; Yong-Mei Liu 0000-0002-0435-0409; Chang-Qin Xin 0000-0003-0635-1066; Xue-Ke Zhao 0000-0002-3032-4933.

Author contributions: Zhao XK designed the study; Huang T performed most of the experiments and wrote the article; all authors contributed to the design and interpretation of the study.

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Tao Huang, Yuan-Qing-Xiao Li, Ming-Yu Zhou, Rui-Han Hu, Gao-Liang Zou, Jian-Chao Li, Shu Feng, Xue-Ke Zhao, Department of Infectious Diseases, Affiliated Hospital of Guizhou Medical University, Guizhou Medical University, Guiyang 550004, Guizhou Province, China

Yong-Mei Liu, Clinical Laboratory Center, Affiliated Hospital of Guizhou Medical University, Guiyang 550004, Guizhou Province, China

Chang-Qin Xin, Department of Infectious Diseases, People's Hospital of Weining Yi, Hui and Miao Autonomous County, Weining 553100, Guizhou Province, China

Corresponding author: Xue-Ke Zhao, PhD, Professor, Department of Infectious Diseases, Affiliated Hospital of Guizhou Medical University, No. 9 Beijing Road, Guiyang 550004, Guizhou Province, China. zhaoxueke1@163.com

Abstract

BACKGROUND

Hepatic stellate cell (HSC) hyperactivation is a central link in liver fibrosis development. HSCs perform aerobic glycolysis to provide energy for their activation. Focal adhesion kinase (FAK) promotes aerobic glycolysis in cancer cells or fibroblasts, while FAK-related non-kinase (FRNK) inhibits FAK phosphorylation and biological functions.

AIM

To elucidate the effect of FRNK on liver fibrosis at the level of aerobic glycolytic metabolism in HSCs.

METHODS

Mouse liver fibrosis models were established by administering CCl_4, and the effect of FRNK on the degree of liver fibrosis in the model was evaluated. Transforming growth factor-β1 was used to activate LX-2 cells. Tyrosine phosphorylation at position 397 (pY397-FAK) was detected to identify activated FAK, and the expression of the glycolysis-related proteins monocarboxylate transporter 1 (MCT-1) and enolase1 (ENO1) was assessed. Bioinformatics analysis was performed to predict putative binding sites for c-myc in the ENO1 promoter region, which were validated with chromatin immunoprecipitation (ChIP) and dual-

interest to declare.

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luciferase reporter assays.

RESULTS
The pY397-FAK level was increased in human fibrotic liver tissue. FRNK knockout promoted liver fibrosis in mouse models. It also increased the activation, migration, proliferation and aerobic glycolysis of primary hepatic stellate cells (pHSCs) but inhibited pHSC apoptosis. Nevertheless, opposite trends for these phenomena were observed after exogenous FRNK treatment in LX-2 cells. Mechanistically, the FAK/Ras/c-myc/ENO1 pathway promoted aerobic glycolysis, which was inhibited by exogenous FRNK.

CONCLUSION
FRNK inhibits aerobic glycolysis in HSCs by inhibiting the FAK/Ras/c-myc/ENO1 pathway, thereby improving liver fibrosis. FRNK might be a potential target for liver fibrosis treatment.

Key Words: Liver fibrosis; Hepatic stellate cells; Focal adhesion kinase; Focal adhesion kinase-related non-kinase; Aerobic glycolysis; Enolase1

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Core Tip: We show that focal adhesion kinase-related non-kinase (FRNK) limits hepatic stellate cell (HSC) activation, proliferation, and migration and promotes HSC apoptosis by inhibiting aerobic glycolysis, thereby ameliorating liver fibrosis. FRNK may represent a potential therapeutic candidate for liver fibrosis treatment.

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INTRODUCTION

Long-term damage to liver function by hepatitis viruses, alcohol, and diet may cause chronic hepatic injuries leading to liver fibrosis and cirrhosis[1-4], which is characterized by the activation of hepatic stellate cells (HSCs) and their transformation into myofibroblasts[5]. This process continuously damages the liver and disrupts the balance of liver self-repair, causing increased cell proliferation and migration and a reduced apoptosis rate[6-8]. At present, the pathological changes associated with chronic liver injuries in individuals without cirrhosis can be reversed after removing the etiological agent, such as in a small proportion of patients with hepatitis B and alcoholic fatty liver disease and most patients with hepatitis C[1,9], but the remaining patients with hepatic fibrosis develop irreversible cirrhosis due to an inability to completely and effectively reverse the pathogenesis and a lack of effective antifibrotic drugs[10]. Therefore, studies of the treatment of liver fibrosis are particularly critical, among which the regulation of the relevant biological functions of HSCs is the most important antifibrotic approach[11,12].

Focal adhesion kinases (FAKs) are a class of nonreceptor cytosolic protein tyrosine kinases that belong to the protein tyrosine kinase superfamily[13,14]. FAK plays an important role in cellular signal transduction and enhances biological behaviors such as proliferation, migration, wound healing and angiogenesis in cells and tissues after integrating signals from integrins, growth factors and mechanical stimuli[15,16]. FAK binds to extracellular matrix (ECM) proteins through an accumulation of integrin receptors to form FAK dimers, which further induce tyrosine phosphorylation at position 397 (pY397-FAK); pY397-FAK regulates these biological functions in cells[17,18] and therefore plays an important role in a variety of malignant tumor cells[18,19]. FAK-related non-kinase (FRNK), which has a nucleotide sequence corresponding to

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the C-terminus of FAK but lacks the N-terminal functional site of FAK, is an independently expressed protein[20] with the main function of inhibiting FAK phosphorylation, thereby inversely regulating the function of FAK after cell activation [21,22]. FRNK negatively regulates FAK signaling axis function, thereby improving pulmonary fibrosis in an experimental mouse model[23].

FAK is also overexpressed in pancreatic ductal adenocarcinoma cells[24,25], promoting the conversion of pyruvate into lactate by increasing enolase1 (ENO1), pyruvate kinase 2, lactate dehydrogenase, and monocarboxylate transporter (MCT)-1 expression and lactate transport, enhancing aerobic glycolysis in cancer cells, and inhibiting mitochondrial oxidative phosphorylation in cancer cells[16]. This switch to aerobic glycolysis is an important mechanism by which tumor cells acquire energy[17,26], as shown by the fact that oxidative phosphorylation simultaneously provides energy to cells performing aerobic glycolysis even in the presence of sufficient oxygen and normal mitochondrial function[27,28]. HSCs also exhibit increased aerobic glycolysis, resulting in lactate accumulation and gluconeogenesis inhibition when they differentiate into myofibroblasts[8,28]. This phenomenon also occurs in individuals with congenital pulmonary fibrosis[29]. Therefore, the inhibition of FAK-related pathways by FRNK may reduce energy acquisition through aerobic glycolysis during HSC activation and could be used as a targeted therapy to ameliorate liver fibrosis. Nevertheless, few studies have focused on the physiological or pathological role of FRNK in obtaining energy during hepatic fibrosis, and its mechanism remains unclear.

In the present study, we first showed that FRNK was downregulated in human liver fibrotic tissues. Then, we verified that FRNK knockout *in vivo* and *in vitro* promoted aerobic glycolysis and hepatic fibrosis. Exogenous FRNK inhibited aerobic glycolysis by inhibiting the FAK/Ras/c-myc/ENO1 pathway, limiting HSC activation, migration, and proliferation and increasing apoptosis to ameliorate liver fibrosis. Together, these data provide a detailed mechanism through which FRNK functions and suggest that FRNK represents a potential target to inhibit aerobic glycolysis in HSCs and treat liver fibrosis.

MATERIALS AND METHODS

Human liver samples

Paraffin blocks of liver tissues from 15 patients with liver fibrosis were collected from the Department of Infectious Diseases, Affiliated Hospital of Guizhou Medical University (Guiyang, China) between March 2019 and September 2019; none of the patients had any other organ-specific or systemic diseases, and liver fibrosis was diagnosed by pathological biopsy. Fifteen healthy liver samples were obtained from distal hepatocarcinoma liver tissue without any abnormalities in specimens surgically resected from patients at the Department of Hepatobiliary Surgery, Affiliated Hospital of Guizhou Medical University. None of the aforementioned subjects had contraindications to liver biopsy, and the study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University (Approval 2018 Ethics Review No. 032) and conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from the patients.

Animals

FRNK knockout ($FRNK^{-/-}$) mice were a gift from the Respiratory and Critical Care Medicine Center, School of Medicine, University of Alabama at Birmingham, Birmingham, AL, United States. All mouse interventions were approved by the Animal Care Committee (IACUC) of Guizhou Medical University (No. 1801109), and the methods and experimental procedures were performed in accordance with the relevant guidelines and regulations. Wild-type (WT) mice of the same genotype were used as controls, and all experimental mice were on the C57BL/6 background.

Mice were maintained under pathogen-free conditions at a controlled temperature (22 ± 2 °C) with a consistent photoperiod (12:12 h light-dark cycle); five mice were housed in each cage, with cages containing soft bedding. The mice were habituated to these conditions for 2 d before inclusion in an experiment. Healthy male mice (aged 8-11 wk, weighing 20 ± 3 g) were selected and intraperitoneally injected with 1.5 μL/g of a 10% Carbon tetrachloride (CCl_4) in corn oil solution three times a week to establish a liver fibrosis model. Mice in the control group were injected with a 1.5 μL/g solution of corn oil three times a week. Livers were harvested at each time point, namely, 0, 2, 4 and 6 wk, for experiments. Six mice per group were used.

Reagents and antibodies

CCl_4, corn oil, and OptiPrep were purchased from Sigma-Aldrich (St. Louis, MO, United States). Transforming growth factor-β1 (TGF-β1) was purchased from R&D Systems (Minneapolis, MN, United States). Primary antibodies specific for the following proteins were purchased from Abcam (Cambridge, United Kingdom): Desmin rabbit monoclonal antibodies (ab32362), FAK rabbit monoclonal antibodies (ab40794), ENO1 mouse monoclonal antibodies (ab190365), alpha SMA rabbit polyclonal antibodies (ab5694), k-ras rabbit monoclonal antibodies (ab275876) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibodies (ab9485). Anti-c-myc (13987) rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Shanghai, China). MCT-1 rabbit polyclonal antibodies (20139-1-AP) were purchased from Proteintech (Wuhan, China). pY397-FAK rabbit polyclonal antibodies (AF3398) were purchased form Affinity Bioscience (Cincinnati, OH, United States). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) and Fisher Scientific (Waltham, MA, United States).

Immunohistochemistry (IHC), hematoxylin & eosin (H&E), Masson's trichrome and Sirius Red staining and hydroxyproline assay

H&E staining kits, Masson's trichrome staining solution and Sirius Red staining solution were purchased from Solarbio Biotechnology Co., Ltd. (Beijing, China) and used according to the manufacturer's guidelines. A hydroxyproline assay was performed using a Nanjing Jiancheng Biotechnology (Nanjing, China) hydroxyproline kit. All kits were used according to the instructions for use. Liver samples were fixed with neutral buffered formalin and embedded in paraffin for IHC. Briefly, sections were incubated with the indicated antibodies. Horseradish peroxidase-conjugated antibodies were used as the secondary antibodies. Finally, a diaminobenzidine colorimetric reagent solution was applied, followed by hematoxylin counterstaining. The slides were then scanned, and representative images were acquired.

Cells and cell culture

LX-2 cells were purchased from Zhongqiao Xinzhou (Shanghai, China). Human HSCs (ZQ0026) and LX-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel). Primary hepatic stellate cells (pHSCs) were extracted from C57BL/6 WT or $FRNK^{-/-}$ mice aged 8-11 wk, as previously described[30,31]. Briefly, the abdominal cavity was opened with a "cross" incision, an 18-gauge trocar was inserted from the left ventricle to inject the preperfusate, and the blood in the liver was flushed by exsanguination until the tissue turned yellow. Then, the preperfusate was replaced with pronase and collagenase for 15-20 min, and the liver was removed and washed with normal saline. The liver capsule and connective tissue were removed, fully digested with a digestion solution at 37 °C with shaking and ground to generate a single-cell suspension. The supernatant was discarded after centrifugation at 1500 rpm for 5 min, and the pellet was resuspended in D-Hank's solution. The hepatocytes were removed by centrifugation, and a gradient lymphocyte separation solution was directly added. HSCs were isolated in one step using monolayer gradient centrifugation, and the cells were washed twice with DMEM and cultured with DMEM containing 10% FBS. Cell survival was evaluated by performing trypan blue staining, and cell purity was identified by desmin immunocytochemical staining. All cells were cultured in an incubator containing 5% CO_2 at 37 °C.

Recombinant FRNK adenoviral vector transfection and HSC activation

An adenovirus-mediated gene delivery system was used to effectively deliver the FRNK cDNA into HSCs. An adenoviral vector carrying the FRNK protein and green fluorescent protein (Ad-FRNK) as well as a green fluorescent protein-carrying adenovirus (Ad-GFP) were purchased from Jikai Gene (Shanghai, China). All transfections were performed according to the manufacturer's instructions, and cells in serum-free medium (DMEM with 1% BSA) were transfected with Ad-FRNK or the control vector (Ad-GFP) 24 h before TGF-β1 treatment. Twenty-four hours later, the cells were cultured with complete medium containing 2 ng/mL TGF-β1 and treated for 36 h[31].

Transwell, cell counting kit-8 (CCK-8) and flow cytometry assays

Transwell migration experiments used 8.0-μm pore size membranes (Corning, United States) according to the manufacturer's protocol. A total of 10^5 cells were seeded in the upper chamber of each well in 100 μL of serum-free medium, while 600 μL of complete

medium was added to the lower chamber as a chemoattractant. After a 6-h incubation at 37 °C, the cells remaining on the upper surface of the membrane were removed with a cotton swab, and the cells on the lower surface of the membrane were considered migrated cells. After fixation with 4% paraformaldehyde and staining with a 0.1% crystal violet solution, images were acquired under an inverted microscope. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Shanghai, China), and 10^4 cells (100 μL/well) were seeded in 96-well plates. After placing the culture plate in an incubator for preincubation (37 °C, 5% CO_2), 10 μL of CCK-8 solution was added to each well, and then the culture plate was evaluated with a microplate reader to detect the absorbance at 450 nm. A total of 10^5 cells in each group were stained with an Annexin V-PE/7-AAD apoptosis kit (Hangzhou Lianke, Hangzhou, Zhejiang Province, China) according to the instructions for use, sorted with a flow cytometer (Beckman, United States) and analyzed using Flow Jo software (Tree Star); dead cells were excluded based on forward scatter and side scatter data.

Western blot analysis

Western blot analysis was performed as previously described[30]. Briefly, 1% NP-40-treated whole-liver tissue lysates or whole-cell lysates were used for Western blot analysis. Protein levels were quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) after total protein extraction. Twenty milligrams of each protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GAPDH was used as a loading control for all blots. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were incubated with primary antibodies overnight at 4 °C. The next day, after an incubation with an appropriate secondary antibody, signals were generated with an electrochemiluminescence detection kit.

Glucose consumption, 2-NBDG uptake and lactate assays

The lactate level in culture medium was detected with the Lactate Colorimetric Assay Kit (BioVision, Milpitas, CA, United States) according to the manufacturer's instructions. The 2-NBDG Glucose Uptake Kit (BioVision, Milpitas, CA, United States) was used to detect the cellular uptake of glucose, and the Glucose Colorimetric Assay Kit (BioVision, Milpitas, CA, United States) was used to detect the glucose concentration in culture medium and thus measure the cellular consumption of glucose. The 2-NBDG Glucose Uptake Kit and the Glucose Colorimetric Assay Kit were used according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP) assay

JASPAR (http://jaspar.genereg.net) and PROMO (http://alggen.lsi.upc.es) database analyses predicted two putative c-myc binding sites in the ENO1 promoter region. A total of 10^7 cells fixed with formaldehyde were collected in 500 μL of lysis buffer from the Magna ChIP HiSens Kit (Millipore, Bedford, Massachusetts, United States) according to the manufacturer's manual. Cells were then sonicated for 25 cycles with a 6-s power-on interval of 30 s and an intensity of 200 W. Afterward, the supernatant was diluted and thoroughly mixed with Protein A/G beads. Then, 5 μg of IgG or an anti-c-myc antibody was added and incubated with the mixture overnight at 4 °C. After washing the beads the next day, the mixture was incubated with elution buffer at 62 °C for 2 h and then 95 °C for 10 min. The eluted DNA was then purified and subjected to a PCR assay to assess the binding sequence. Specific primer sequences were used to perform PCR.

Dual-luciferase reporter assays

The effect of c-myc on the ENO1 promoter was determined by cotransfecting pcDNA-c-myc or pcDNA-vector (NC) into LX-2 cells with pGL3-based constructs containing an empty sequence (NC) or the WT or MT1/MT2 ENO1 promoter sequences, and Renilla luciferase reporter plasmids. Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured with a luciferase reporter assay kit (Genomeditech, Shanghai, China). Fluorescence detection was performed according to the instructions of the instrument, the parameters were set, the measurement time was 10 s, and the measurement interval was 2 s. Each sample was added into a measuring tube in a total volume of 20 μL (the sample volume was consistent in each measurement), and then 20 μL of Firefly Luciferase Assay Reagent was added, mixed well 2-3 times (without vortexing), mixed well again and evaluated to determine relative light unit (RLU) 1. Cell lysis buffer was set as the blank control well. The tested samples were mixed with 20 μL of prepared Renilla Luciferase Assay working solution

2-3 times and mixed well before measuring RLU2. The measured RLU1 value was compared to the corresponding RLU2 value, and the resulting ratio determined the degree of reporter activation. The ratio of firefly luciferase activity to Renilla luciferase activity was calculated for each sample.

Statistical analysis

Data were statistically analyzed using GraphPad Prism 5.0 software, and a two-tailed Student's *t* test was used for comparisons between different groups. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SD.

RESULTS

The level of the pY397-FAK protein was increased while the level of the FRNK protein was decreased in human fibrotic liver tissue

We first investigated the level of pY397-FAK in fibrotic liver tissue to explore the role of pY397-FAK in liver fibrosis. Compared with normal liver tissue, liver tissue samples from patients in the liver fibrosis group showed typical pathological features, including significant steatosis, inflammatory necrosis, significant collagen deposition, hepatic fibrosis and hepatocyte loosening. Masson's trichrome staining showed less collagen deposition and a normal cell morphology in the normal group, while a large number of blue-stained collagen fibers was observed in the liver fibrosis group, and the tissue had accumulated a wide band of collagen fibers that extended into and was distributed in the hepatic lobules. Sirius Red staining showed less collagen deposition in normal subjects and a normal cell morphology but substantial red staining indicating collagen deposition in portal areas in fibrotic liver tissues. Notably, IHC showed higher α-smooth muscle actin (α-SMA) and pY397-FAK expression in fibrotic liver tissue than in normal liver tissues (Figure 1A and B). Western blot analysis showed higher levels of the pY397-FAK protein in fibrotic liver tissues than in normal liver tissues. Conversely, in fibrotic liver tissue, FRNK was expressed at lower levels than that in normal tissues ($P < 0.05$, Figure 1C and D). These results suggest that pY397-FAK protein expression is increased and FRNK protein expression is decreased in fibrotic liver tissue.

Exacerbation of liver fibrosis and aerobic glycolysis in mice after FRNK knockout in vivo

We established a fibrosis model by injecting CCl_4 into WT mice and FRNK$^{-/-}$ mice. After two fortnights, the expression of the pY397-FAK protein peaked, while the FRNK protein was expressed at a low level ($P < 0.05$, Figure 2A and B). Therefore, mouse models with four weeks of injection were used in subsequent experiments. By performing H&E, Masson's trichrome and Sirius Red staining, we found a greater liver fibrosis area and more extensive liver fibrosis in FRNK$^{-/-}$ mice than in WT mice after the CCl_4 intervention ($P < 0.05$, Figure 2C and D), while the hydroxyproline content in FRNK$^{-/-}$ mice with fibrosis was greater than that in WT mice with fibrosis ($P < 0.05$, Figure 2E). Western blot analysis revealed higher levels of the pY397-FAK, MCT-1, ENO1 and α-SMA proteins in the liver tissues from FRNK$^{-/-}$ mice treated with CCl_4 than in WT mice ($P < 0.05$, Figure 2F and G). Based on these results, FRNK$^{-/-}$ mice develop more severe liver fibrosis after the CCl_4 intervention, along with increased expression of the aerobic glycolysis-related proteins MCT-1 and ENO1. It suggests that there may be more active aerobic glycolysis in the liver.

FRNK knockout promotes liver fibrosis and aerobic glycolysis in vitro

We extracted pHSCs from WT mice and FRNK$^{-/-}$ mice for *in vitro* experiments (Supplementary Figure 1). After 36 h of TGF-β1 treatment, the migration of pHSCs from the FRNK$^{-/-}$ groups in a Transwell chamber was increased ($P < 0.05$, Figure 3A). As indicated by the level of cell proliferation, pHSCs from the FRNK$^{-/-}$ group exhibited increased cellular activity ($P < 0.05$, Figure 3B). After adding TGF-β1 to pHSCs from FRNK$^{-/-}$ mice for 36 h, the apoptosis rate was lower than that of pHSCs from the control mice ($P < 0.05$, Figure 3C). Moreover, pHSCs from FRNK$^{-/-}$ mice showed increased glucose uptake and consumption compared with control pHSCs. Additionally, the lactate level in the medium of pHSCs from FRNK$^{-/-}$ mice was increased compared with the lactate level in the medium of pHSCs from the control group ($P < 0.05$, Figure 3D and E). Western blot analysis showed higher levels of the MCT-1, ENO1 and α-SMA proteins in the pHSCs from FRNK$^{-/-}$ mice was higher than in the

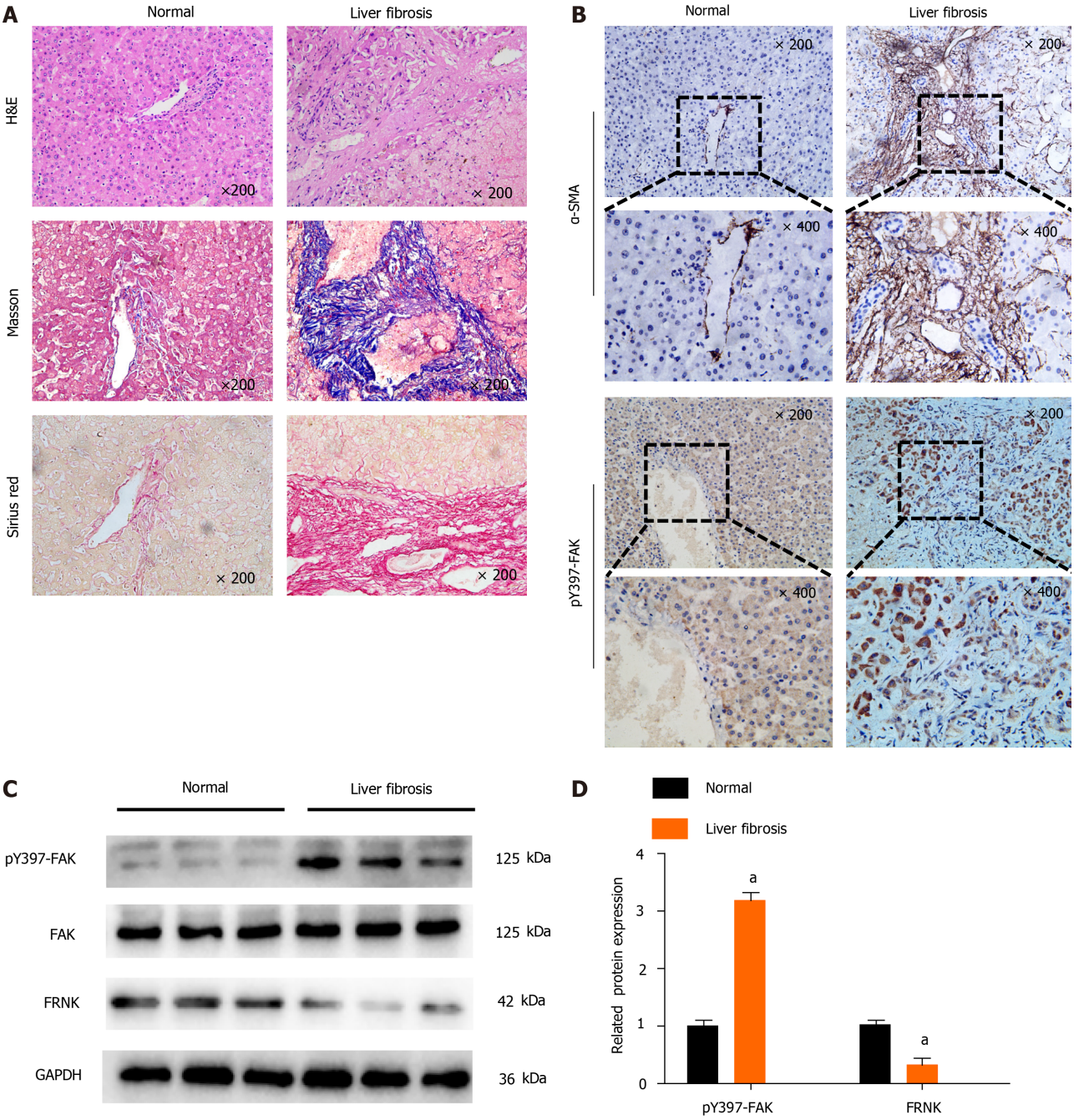
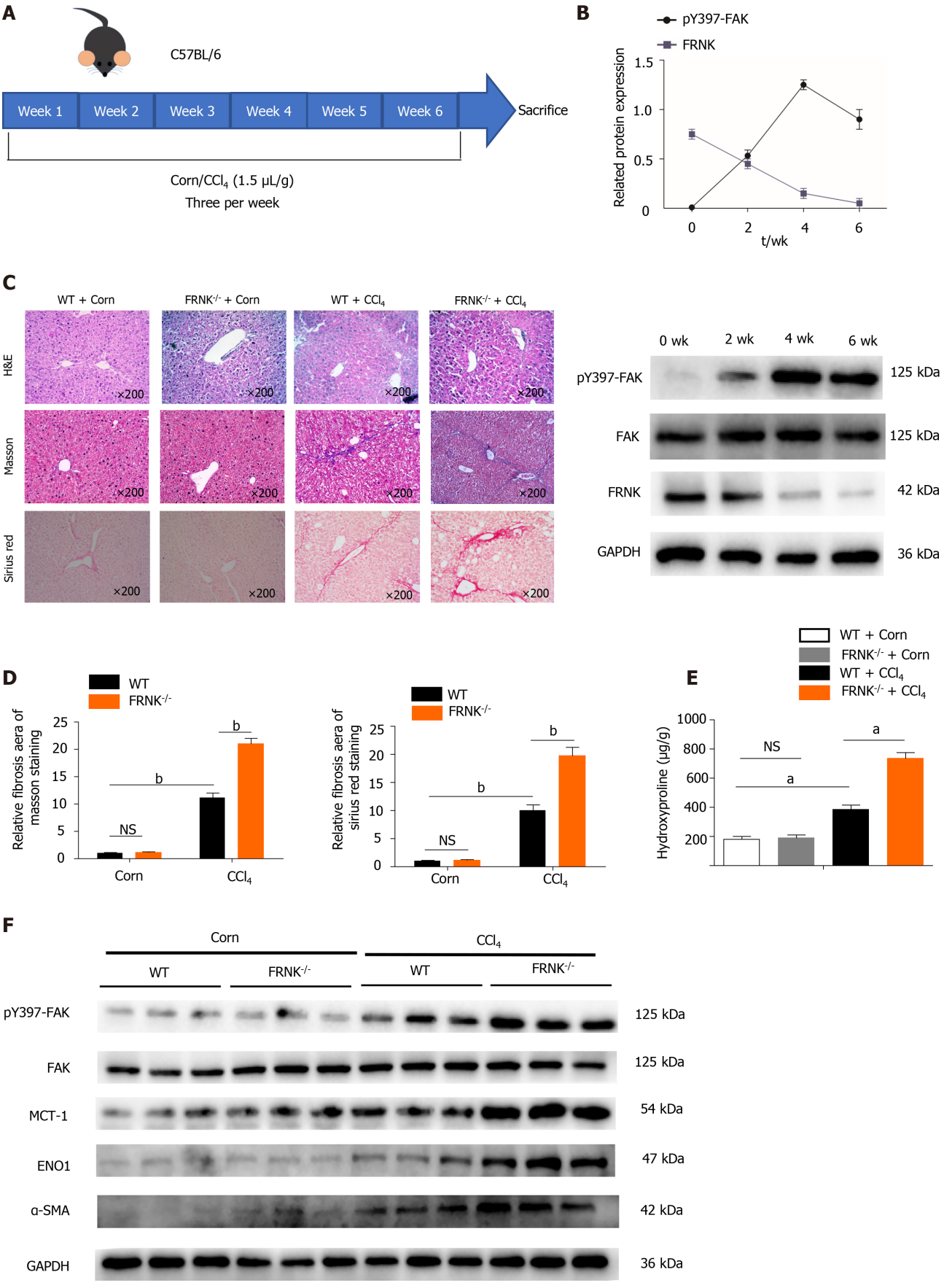


Figure 1 Tyrosine phosphorylation at position 397 of FAK is upregulated, while FRNK expression is downregulated in human fibrotic liver tissue. A: H&E, Masson's trichrome and Sirius Red staining were performed after liver biopsy to assess the tissues of normal subjects and patients with liver fibrosis under a light microscope at 200× magnification; B: Immunohistochemistry showed changes in the expression of α-smooth muscle actin (α-SMA) and tyrosine phosphorylation at position 397 of FAK(pY397-FAK) in the livers of normal subjects compared with patients with liver fibrosis under a light microscope at 200× or 400× magnification; C and D: Protein expression in biopsy tissues was analyzed using Western blotting. Representative results from three independent replicate assays are shown. $^{a}P < 0.05$. Data are presented as the mean ± SD. FAK: Focal adhesion kinase; FRNK: Focal adhesion kinase-related non-kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

pHSCs from WT mice ($P < 0.05$, Figure 3F and G). The above results illustrate that FRNK knockout in mice increases the activation, migration, and proliferation of pHSCs and attenuates pHSC apoptosis while enhancing their aerobic glycolytic capacity *in vitro*.

Exogenous FRNK ameliorates experimental liver fibrosis and aerobic glycolysis in vitro

We transfected LX-2 cells with an adenovirus containing FRNK, induced the expression of the exogenous FRNK gene and incubated the cells with TGF-β1 for 36 h. We then performed Transwell, CCK-8 and flow cytometry assays with the transfected


A
C57BL/6
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Sacrifice
Corn/CCl4 (1.5 μL/g)
Three per week
B
pY397-FAK
FRNK
Related protein expression
t/wk
C
WT + Corn
FRNK-/- + Corn
WT + CCl4
FRNK-/- + CCl4
H&E
Masson
Sirius red
×200
0 wk
2 wk
4 wk
6 wk
pY397-FAK
FAK
FRNK
GAPDH
125 kDa
125 kDa
42 kDa
36 kDa
D
WT
FRNK-/-
Relative fibrosis aera of masson staining
Relative fibrosis aera of sirius red staining
NS
b
Corn
CCl4
E
Hydroxyproline (μg/g)
NS
a
F
Corn
CCl4
WT
FRNK-/-
pY397-FAK
FAK
MCT-1
ENO1
α-SMA
GAPDH
125 kDa
125 kDa
54 kDa
47 kDa
42 kDa
36 kDa

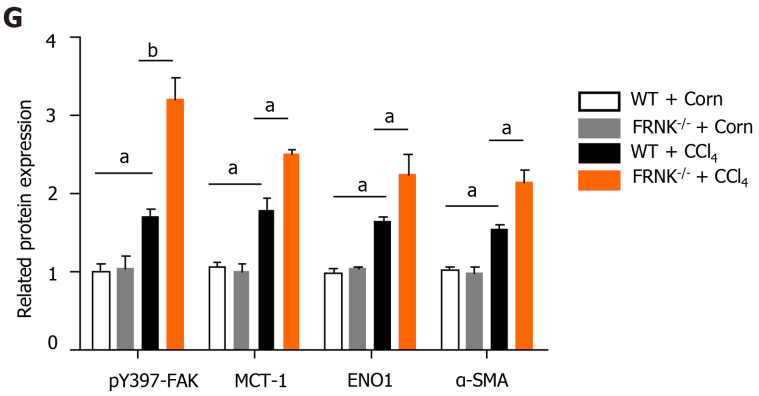


Figure 2 Liver fibrosis in mice was aggravated after FRNK knockout. A and B: WT mice were modeled for 6 wk, pY397-FAK and FRNK protein expression levels *in vivo* was measured using Western blotting every fortnight; C and D: FRNK$^{-/-}$ and WT mice were used to establish a liver fibrosis model by administering CCl_4 (1.5 μL/g), and liver tissues from these mice were stained with H&E, Masson's trichrome, and Sirius Red after 4 wk and observed under a light microscope × 200 magnification. The relative fibrotic areas were analyzed; E: The hydroxyproline content in liver tissues from the liver fibrosis model was also measured; F and G: Western blotting was used to detect the relative expression of proteins in the liver fibrosis model established with FRNK$^{-/-}$ mice and WT mice. Representative results from three independent replicate assays are shown (n = 6). $^{a}P < 0.05$ and $^{b}P < 0.01$. Data are presented as the mean ± SD. MCT-1: Monocarboxylate transporter-1; ENO1: Enolase1.

cells to evaluate migration, proliferation and apoptosis. The migration of LX-2 cells was inhibited and apoptosis was increased after the introduction of exogenous FRNK compared to the control treatment ($P < 0.05$, Figure 4A and C). In addition, proliferation was also inhibited ($P < 0.01$, Figure 4B). Based on these results, exogenous FRNK inhibits cell migration and proliferation and promotes apoptosis. The abilities of cells in the Ad-FRNK group to take up and consume glucose were reduced, and the lactate level in the cell culture medium was reduced ($P < 0.05$, Figure 4D and E). Subsequently, cellular proteins were extracted, and the relative levels of intracellular pY397-FAK, MCT-1, ENO1 and α-SMA proteins were detected by Western blotting. The relative expression of the aforementioned proteins in the Ad-FRNK group was lower than that in the control group ($P < 0.05$, Figure 4F and G). Thus, the introduction of exogenous FRNK into HSCs inhibits cell proliferation and migration and promotes apoptosis. It also inhibits cellular aerobic glycolysis and thus inhibits cellular energy generation *in vitro*.

FRNK does not directly target ENO1

To explore the precise molecular mechanism by which FRNK regulates the ENO1 protein, TGF-β1 was used to stimulate LX-2 cells to activate FAK. The level of pY397-FAK was increased after stimulation with TGF-β1. The expression of the K-Ras, c-myc and ENO1 proteins downstream of FAK was also examined ($P < 0.05$, Figure 5A and B). While examining whether FRNK directly inhibits ENO1 protein expression, increased ENO1 protein expression was observed after introducing exogenous c-myc into LX-2 cells, but ENO1 protein expression was not reduced after the continued introduction of exogenous FRNK ($P < 0.05$, Figure 5C and D), suggesting that exogenous FRNK does not directly inhibit ENO1 protein expression to exert its biological function. As a method to investigate whether c-myc directly regulates ENO1, a bioinformatics analysis of the ENO1 promoter was performed to predict the putative binding site for c-myc in the ENO1 promoter, followed by ChIP and dual-luciferase reporter assays to verify that c-myc transcriptionally activates the ENO1 promoter ($P < 0.05$, Figure 5E and F). The results suggested that TGF-β1 activates FAK by inducing FAK phosphorylation at position 397. Then, pY397-FAK increased the expression of downstream K-Ras and c-myc proteins, followed by transcriptional activation of ENO1 expression by c-myc, promoting aerobic glycolysis and activation in HSCs. pY397-FAK and its biological functions were inhibited by the introduction of exogenous FRNK, limiting aerobic glycolysis and activation in HSCs and ameliorating liver fibrosis (Figure 6).

DISCUSSION

The imbalance between liver injury and self-repair is the key to the development of liver fibrosis, and restoring the balance from an imbalanced state is a potential

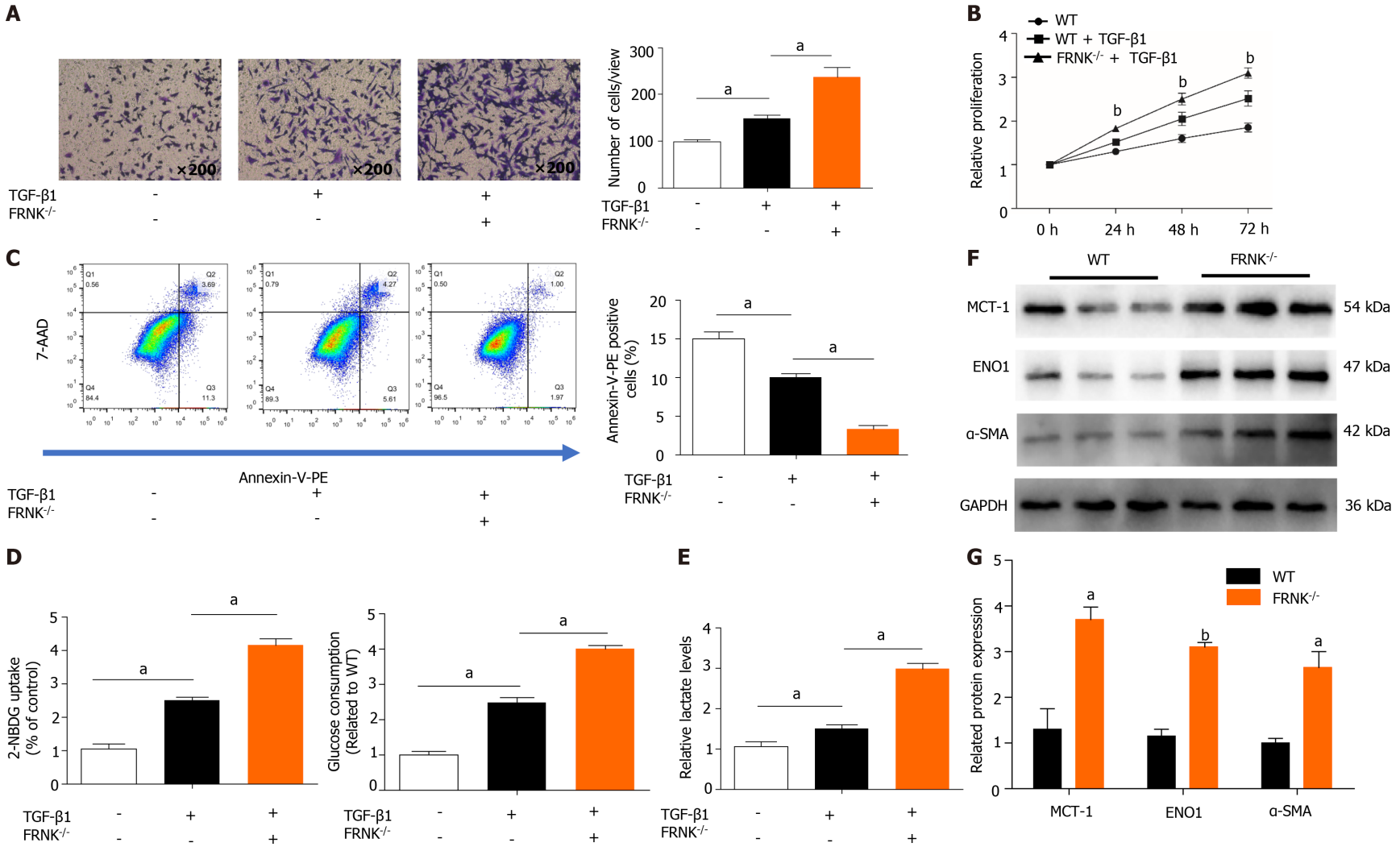


Figure 3 Knockout of FRNK promotes liver fibrosis and aerobic glycolysis *in vitro*. A: After 36 h of culture with TGF-β1 (2 ng/mL), the migratory ability of primary hepatic stellate cells(pHSCs) was measured under a light microscope at × 200 magnification (10^5 cells per well); B: The proliferation of pHSCs was assessed with a CCK-8 assay; C: The apoptosis of pHSCs was analyzed using flow cytometry after 36 h of intervention; D and E: pHSCs cultured under the same intervention conditions were examined for glucose uptake and consumption, and lactate levels in the cell culture medium were also assessed; F and G: MCT-1, ENO1 and α-SMA levels in pHSCs were assessed using Western blotting. Representative results from three independent replicate assays are shown. $^aP < 0.05$ and $^bP < 0.01$ Results are presented as the mean ± SD.

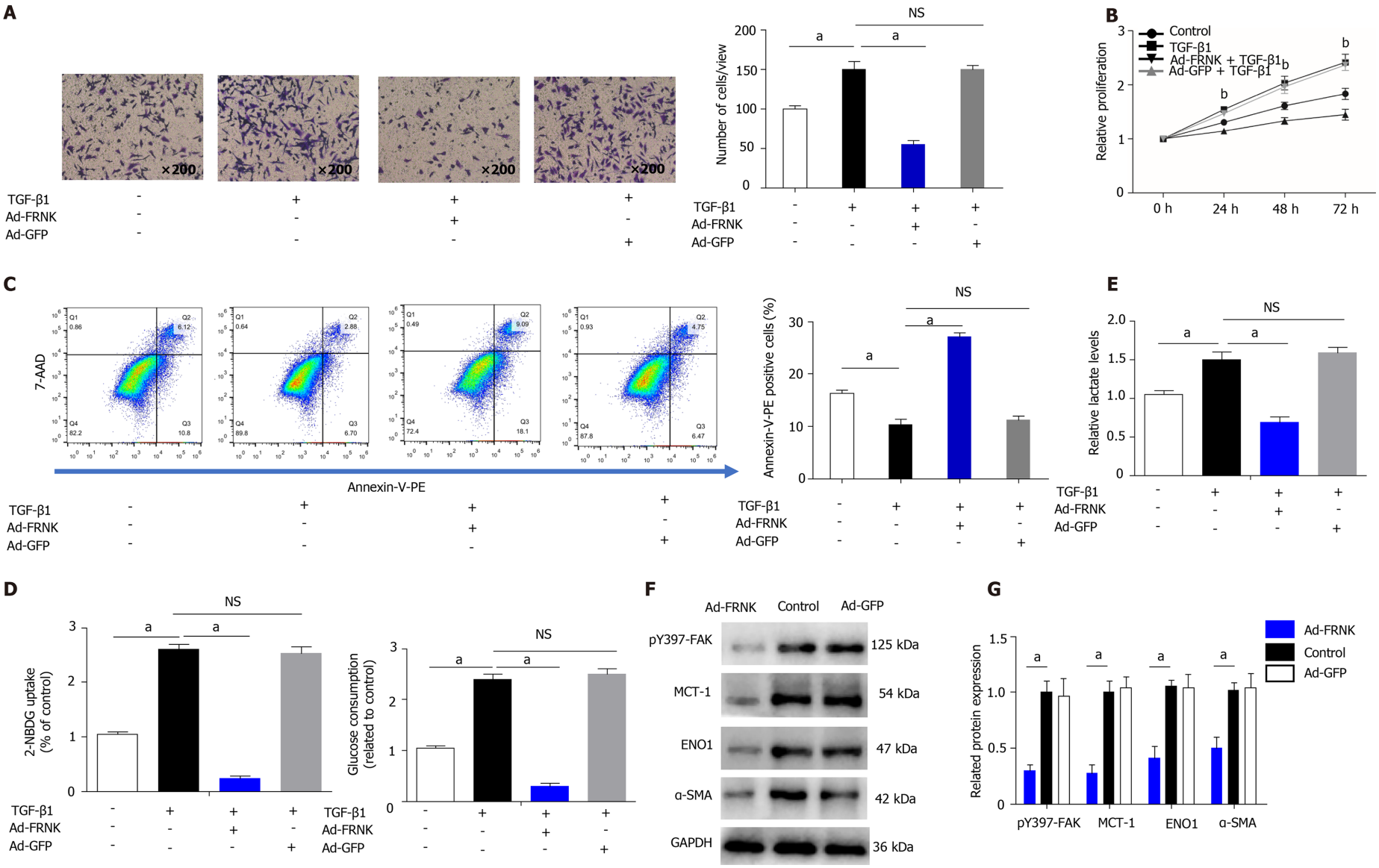


Figure 4 Exogenous FRNK ameliorates experimental liver fibrosis and aerobic glycolysis *in vitro*. LX-2 cells were transfected with a green fluorescent protein-carrying adenoviral vector (Ad-GFP) also encoding the FRNK gene. A: Cultured with TGF-β1 (2 ng/mL) for 36 h (10^5 cells per well). Migration was measured by analyzing cells under a light microscope × 200 magnification; B: The proliferation of LX-2 cells cultured under the intervention conditions is presented; C: The

apoptosis of LX-2 cells was analyzed after 36 h of intervention using flow cytometry; D and E: LX-2 cells cultured under the same intervention conditions were examined for glucose consumption and uptake abilities, and lactate levels in the cell culture medium were also assessed; F and G: Levels of pY397-FAK, MCT-1, ENO1 and α-SMA in LX-2 cells were detected using Western blotting. Representative results from three independent replicate assays are shown . $^{a}P < 0.05$ and $^{b}P < 0.01$. Results are presented as the mean ± SD.

treatment for liver diseases. In the present study, we verified that FRNK alters the activation, proliferation, migration and apoptosis of HSCs by regulating aerobic glycolysis during liver fibrosis. FRNK inhibits aerobic glycolysis in HSCs by suppressing ENO1 activation through the FAK/Ras/c-myc/ENO1 pathway.

Early studies by our group verified that FAK plays important roles in the activation of HSCs and the development of liver fibrosis and that inhibition of FAK gene expression inhibits liver fibrogenesis[30]. Ding *et al*[23] verified that FRNK negatively regulates pulmonary fibrosis induced by FAK phosphorylation during pulmonary fibrosis. If FRNK inhibits the biological function of FAK in pulmonary fibrosis and uses a similar mechanism to repress liver fibrosis, it may represent a potential therapeutic target in liver fibrosis. Previous studies on FRNK have focused on the inhibition of the migratory function of vascular smooth muscle[32,33], combined with the presence of extracellular lactate accumulation during HSC activation[28,34] and FAK activation of aerobic glycolytic function in tumor cells[35-37]. FRNK may improve liver fibrosis by inhibiting aerobic glycolysis and inhibiting FAK activation in HSCs, but the mechanism by which FRNK exerts this effect remains unclear.

In the current study, we first observed increased expression of the pY397-FAK protein and decreased expression of the FRNK protein in tissues from patients with liver fibrosis (Figure 1). Subsequent experiments using CCl_4 to replicate liver fibrosis in a mouse model yielded the same results (Figure 2B). Therefore, we speculated that a correlation between the occurrence of liver fibrosis and the downregulation of FRNK expression may exist and subsequently performed experiments in WT mice and FRNK$^{-/-}$ mice. After the CCl_4 intervention, the degree of liver fibrosis in WT mice was lower than that in FRNK$^{-/-}$ mice (Figure 2C and D). The expression of the aerobic glycolysis-related proteins MCT-1 and ENO1 in the liver tissue of FRNK$^{-/-}$ mice was increased (Figure 2F and G), suggesting that FRNK gene deletion may promote intrahepatic aerobic glycolysis and aggravate the occurrence and development of liver fibrosis. We extracted pHSCs from WT mice and FRNK$^{-/-}$ mice for *in vitro* experiments to further explore the effect of FRNK on the biological functions of HSCs. After treatment with TGF-β1, the biological functions of pHSCs from FRNK$^{-/-}$ mice were more active than those of pHSCs from WT mice, as evidenced by the increased migration and proliferation and reduced apoptosis rate (Figure 3A-C). Furthermore, the uptake and consumption of glucose and extracellular lactate levels of FRNK$^{-/-}$ pHSCs were increased (Figure 3D and E), suggesting that HSCs lacking FRNK exhibited more active aerobic glycolysis, which supplied energy for their biological functions, such as activation, proliferation and migration. On the other hand, we transfected LX-2 cells with an adenovirus carrying the FRNK gene to verify whether exogenous FRNK is a promising therapeutic target in liver fibrosis. After FRNK

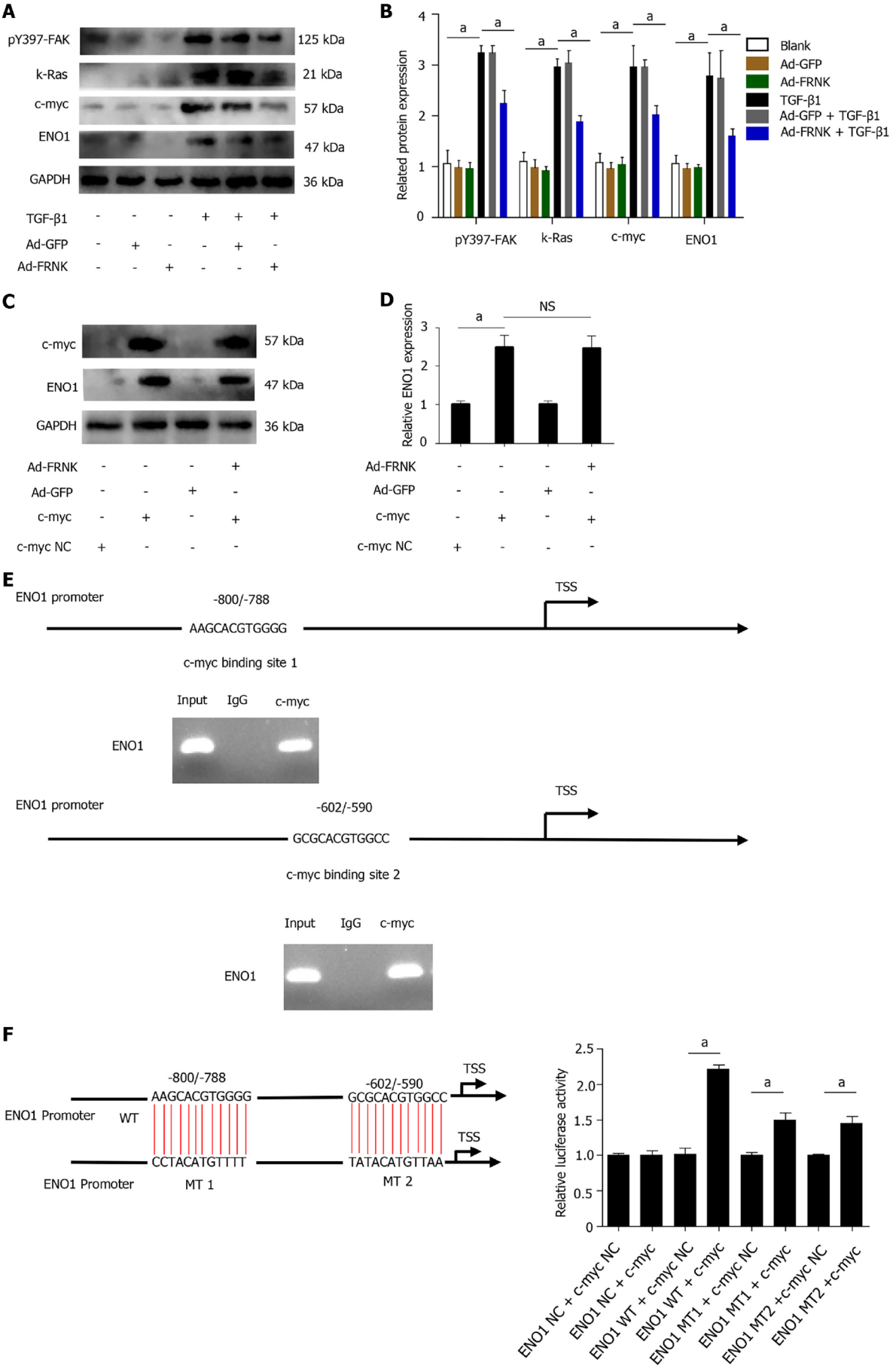


Figure 5 FRNK does not directly target ENO1. A and B: Proteins were extracted from LX-2 cells, and relative levels of the pY397-FAK, K-Ras, c-myc and

ENO1 proteins were determined; C and D: After transfection of LX-2 cells with pcDNA-c-myc or pcDNA-vector (NC), adenoviral vectors containing the FRNK gene (Ad-FRNK) or the negative control (Ad-GFP) were transfected, and the extracted protein was used to evaluate ENO1 expression through Western blotting; E: Schematic representation of the structure of the putative c-myc binding site in the human ENO1 promoter and chromatin immunoprecipitation (ChIP) assays with anti-c-myc or IgG; F: A dual-luciferase reporter assay showed the luciferase activity of WT, mutation (MT)1 and MT2 ENO1 promoters in LX-2 cells transfected with the c-myc or NC plasmid. Representative results from three independent replicate assays are shown. $^{a}P < 0.05$ and $^{b}P < 0.01$. Results are presented as the mean ± SD.

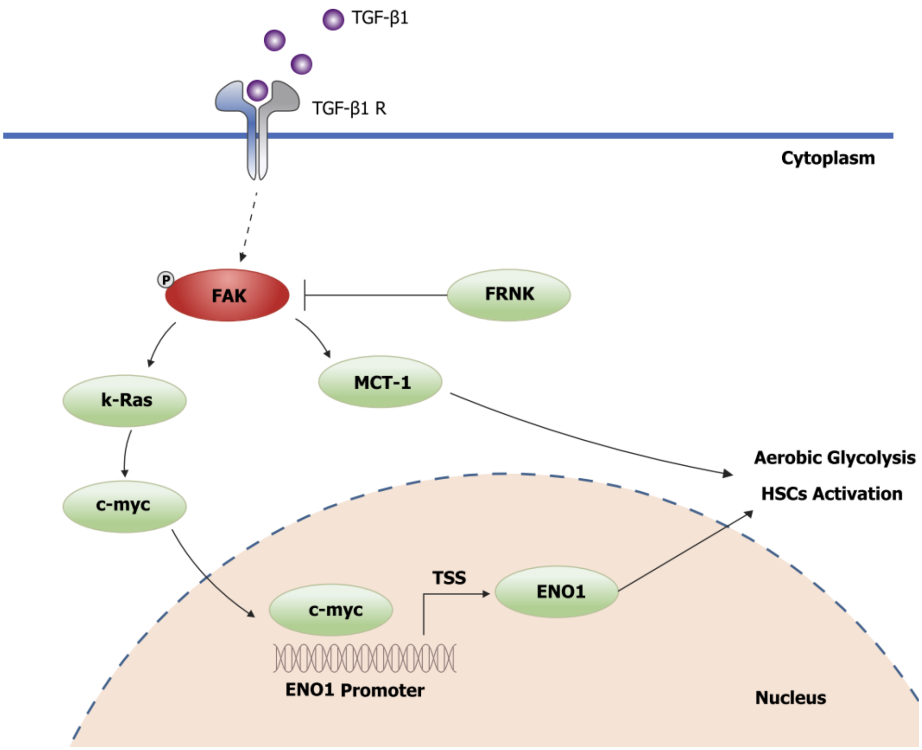


Figure 6 Schematic diagram of FRNK inhibition of the FAK/Ras/c-myc/ENO1 pathway to ameliorate liver fibrosis.

overexpression, cell proliferation and migration decreased, while the percentage of apoptotic cells increased (Figure 4A-C). The uptake and utilization of glucose and the extracellular lactate level in LX-2 cells were also decreased (Figure 4D and E), and pY397-FAK expression in these cells was decreased (Figure 4F and G). These results further indicated that increasing exogenous FRNK expression prevented HSCs from obtaining energy through aerobic glycolysis and reduced cell activation and the energy supply required for a series of biological functions after activation, thereby inhibiting liver fibrosis. While investigating the mechanism by which FRNK regulates aerobic glycolysis in HSCs, we found that FAK is phosphorylated in LX-2 cells stimulated with TGF-β1 and that the downstream proteins K-Ras, c-myc, and ENO1 are activated. Following the introduction of FRNK, ENO1 protein expression was reduced (Figure 5A and B). We transfected both the c-myc and FRNK genes into LX-2 cells to determine whether FRNK directly inhibited ENO1 expression and found that ENO1 protein expression was not reduced upon increased exogenous FRNK expression (Figure 5C and D), thus suggesting that FRNK does not directly inhibit ENO1 protein expression. Therefore, we further hypothesized that c-myc directly alters ENO1 protein expression. Early studies revealed that c-myc is involved in the regulation of various biological functions, including metabolism, cell growth, cell cycle regulation and apoptosis[38,39], consistent with the results of our study. An increasing number of studies have shown that c-myc is involved in the regulation of promoters as a transcription factor. Hence, we predicted the putative c-myc binding site in the ENO1 promoter region by performing a bioinformatics analysis, followed by confirmation of our hypothesis that c-myc transcriptionally activates ENO1 and subsequently promotes liver fibrosis in HSCs by performing dual-luciferase reporter and ChIP assays (Figure 5E and F). Therefore, our study revealed that FRNK alleviated hepatic fibrosis *via* the FAK/Ras/c-myc/ENO1 pathway. The molecular mechanism by which FRNK regulates ENO1 and MCT-1 expression should be confirmed by conducting more complicated investigations in the future, and our group will be

dedicated to studying this pathway.

CONCLUSION

In conclusion, this study is the first to reveal the effect of FRNK on liver fibrosis at the metabolic level. The experimental results suggest that the FAK/FRNK genes are potentially useful therapeutic targets in liver fibrosis and provide some rationale for the development of related drugs in the future.

ARTICLE HIGHLIGHTS

Research background

Hepatic stellate cell (HSC) hyperactivation is a central link in liver fibrosis development. HSCs perform aerobic glycolysis to provide energy for their activation.

Research motivation

Focal adhesion kinase (FAK) promotes aerobic glycolysis in cancer cells or fibroblasts, while FAK-related non-kinase (FRNK) inhibits FAK phosphorylation and biological functions.

Research objectives

To elucidate the effect of FRNK on liver fibrosis at the level of aerobic glycolytic metabolism in HSCs.

Research methods

Mouse liver fibrosis models were established by administering CCl_4, and the effect of FRNK on the degree of liver fibrosis in the model was evaluated. Transforming growth factor-β1 was used to activate LX-2 cells. Tyrosine phosphorylation at position 397 (pY397-FAK) was detected to identify activated FAK, and the expression of the glycolysis-related proteins monocarboxylate transporter 1 (MCT-1) and enolase1 (ENO1) was assessed. Bioinformatics analysis was performed to predict putative binding sites for c-myc in the ENO1 promoter region, which were validated with chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays.

Research results

The pY397-FAK level was increased in human fibrotic liver tissue. FRNK knockout promoted liver fibrosis in mouse models. It also increased the activation, migration, proliferation and aerobic glycolysis of primary hepatic stellate cells (pHSCs) but inhibited pHSC apoptosis. Nevertheless, opposite trends for these phenomena were observed after exogenous FRNK treatment in LX-2 cells. Mechanistically, the FAK/Ras/c-myc/ENO1 pathway promoted aerobic glycolysis, which was inhibited by exogenous FRNK.

Research conclusions

FRNK inhibits aerobic glycolysis in HSCs by inhibiting the FAK/Ras/c-myc/ENO1 pathway, thereby improving liver fibrosis. FRNK might be a potential target for liver fibrosis treatment.

Research perspectives

The molecular mechanism by which FRNK regulates ENO1 and MCT-1 expression should be confirmed by conducting more complicated investigations in the future, and our group will be dedicated to studying this pathway.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Dynamics of cytokines predicts risk of hepatocellular carcinoma among chronic hepatitis C patients after viral eradication

Ming-Ying Lu, Ming-Lun Yeh, Ching-I Huang, Shu-Chi Wang, Yi-Shan Tsai, Pei-Chien Tsai, Yu-Min Ko, Ching-Chih Lin, Kuan-Yu Chen, Yu-Ju Wei, Po-Yao Hsu, Cheng-Ting Hsu, Tyng-Yuan Jang, Ta-Wei Liu, Po-Cheng Liang, Ming-Yen Hsieh, Zu-Yau Lin, Shinn-Cherng Chen, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Ming-Lung Yu

ORCID number: Ming-Ying Lu 0000-0002-1317-9586; Ming-Lun Yeh 0000-0003-3728-7618; Ching-I Huang 0000-0002-5467-0398; Shu-Chi Wang 0000-0001-9691-7507; Yi-Shan Tsai 0000-0002-5251-1318; Pei-Chien Tsai 0000-0002-5044-6727; Yu-Min Ko 0000-0002-8424-9289; Ching-Chih Lin 0000-0002-4829-308X; Kuan-Yu Chen 0000-0001-9350-2699; Yu-Ju Wei 0000-0003-1266-7796; Po-Yao Hsu 0000-0002-5443-7203; Cheng-Ting Hsu 0000-0002-9057-3536; Tyng-Yuan Jang 0000-0003-2961-130X; Ta-Wei Liu 0000-0002-6978-9922; Po-Cheng Liang 0000-0001-9189-6604; Ming-Yen Hsieh 0000-0002-8019-3011; Zu-Yau Lin 0000-0002-8489-7147; Shinn-Cherng Chen 0000-0002-5925-4078; Chung-Feng Huang 0000-0002-3367-068X; Jee-Fu Huang 0000-0002-2752-7051; Chia-Yen Dai 0000-0003-2296-3054; Wan-Long Chuang 0000-0002-2376-421X; Ming-Lung Yu 0000-0001-8145-1900.

Author contributions: Lu MY analyzed the data and wrote the manuscript; Tsai PC confirmed the statistical analysis; Wang SC, Tsai YS, Ko YM, Lin CC, and Chen KY performed the experiments; Yeh ML, Huang CI, Wei YJ, Hsu PY, Hsu CT, Jang TY, Liu TW, Liang PC, Hsieh MY, Lin ZY, Chen SC, Huang CF, Huang JF, and Dai CY

Ming-Ying Lu, Ming-Lun Yeh, Ching-I Huang, Yi-Shan Tsai, Pei-Chien Tsai, Yu-Min Ko, Ching-Chih Lin, Kuan-Yu Chen, Yu-Ju Wei, Po-Yao Hsu, Cheng-Ting Hsu, Tyng-Yuan Jang, Ta-Wei Liu, Po-Cheng Liang, Ming-Yen Hsieh, Zu-Yau Lin, Shinn-Cherng Chen, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Ming-Lung Yu, Hepatitis Center and Hepatobiliary Division, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

Ming-Lun Yeh, Ching-I Huang, Zu-Yau Lin, Shinn-Cherng Chen, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Ming-Lung Yu, School of Medicine and Hepatitis Research Center, College of Medicine, Center for Cancer Research and Center for Liquid Biopsy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

Shu-Chi Wang, Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

Chia-Yen Dai, Health Management Center, Department of Community Medicine, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

Ming-Lung Yu, Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 80708, Taiwan

Corresponding author: Ming-Lung Yu, MD, PhD, Chief Doctor, Full Professor, Hepatitis Center and Hepatobiliary Division, Department of Internal Medicine, Kaohsiung Medical University Hospital, No. 100 Shih-Chuan 1st Road, Sanmin District, Kaohsiung 80708, Taiwan. fish6069@gmail.com

Abstract

BACKGROUND

Chronic hepatitis C virus (HCV) infection induces profound alterations in the cytokine and chemokine signatures in peripheral blood. Clearance of HCV by antivirals results in host immune modification, which may interfere with immune-mediated cancer surveillance. Identifying HCV patients who remain at risk of hepatocellular carcinoma (HCC) following HCV eradication remains an unmet need. We hypothesized that antiviral therapy-induced immune reconstruc-

collected the clinical data; Yu ML and Chuang WL designed the study, interpreted data, and supervised the manuscript; all authors had read and approved the final manuscript.

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tion may be relevant to HCC development.

AIM
To investigate the impact of differential dynamics of cytokine expression on the development of HCC following successful antiviral therapy.

METHODS
One hundred treatment-naïve HCV patients with advanced fibrosis (F3/4) treated with direct-acting antivirals (DAAs) or peginterferon/ribavirin who achieved sustained virologic response [SVR, defined as undetectable HCV RNA throughout 12 wk (SVR12) for the DAA group or 24 wk (SVR24) for the interferon group after completion of antiviral therapy] were enrolled since 2003. The primary endpoint was the development of new-onset HCC. Standard HCC surveillance (abdominal ultrasound and α-fetoprotein) was performed every six months during the follow-up. Overall, 64 serum cytokines were detected by the multiplex immunoassay at baseline and 24 wk after end-of-treatment.

RESULTS
HCC developed in 12 of the 97 patients over 459 person-years after HCV eradication. In univariate analysis, the Fibrosis-4 index (FIB-4), hemoglobin A1c (HbA1c), the dynamics of tumor necrosis factor-α (TNF-α), and TNF-like weak inducer of apoptosis (TWEAK) after antiviral therapy were significant HCC predictors. The multivariate Cox regression model showed that ΔTNF-α (≤ -5.7 pg/mL) was the most important risk factor for HCC (HR = 11.54, 95%CI: 2.27-58.72, P = 0.003 in overall cases; HR = 9.98, 95%CI: 1.88-52.87, P = 0.007 in the interferon group). An HCC predictive model comprising FIB-4, HbA1c, ΔTNF-α, and ΔTWEAK had excellent performance, with 3-, 5-, 10-, and 13-year areas under the curve of 0.882, 0.864, 0.903, and 1.000, respectively. The 5-year accumulative risks of HCC were 0%, 16.9%, and 40.0% in the low-, intermediate-, and high-risk groups, respectively.

CONCLUSION
Downregulation of serum TNF-α significantly increases the risk of HCC after HCV eradication. A predictive model consisting of cytokine kinetics could ameliorate personalized HCC surveillance strategies for post-SVR HCV patients.

Key Words: Hepatitis C virus; Hepatocellular carcinoma; Sustained virologic response; Tumor necrosis factor-α; Tumor necrosis factor-like weak inducer of apoptosis; Cytokine

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Core Tip: Successful hepatitis C virus (HCV) eradication does not eliminate hepatocellular carcinoma (HCC) development. Clearance of HCV by antiviral agents results in host immune modification, which might interfere with immune-mediated cancer surveillance. We attempted to identify immune biomarkers to predict HCC occurrence after antiviral therapy. The dynamics of serum tumor necrosis factor-α (TNF-α) and TNF-like weak inducer of apoptosis were associated with HCC occurrence after HCV clearance. We established a predictive model to assess the risk of HCC among HCV patients after HCV eradication. Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC). As HCV treatment evolves from an interferon (IFN)-based regimen to a therapy based on direct-acting antiviral agents (DAAs), it yields a sustained virologic response (SVR) rate of more than 97% in chronic hepatitis C patients[1,2]. However, successful antiviral therapy does not eliminate HCC development. In a meta-analysis of observational studies, IFN therapy decreased the risk of HCC by 76% in patients with bridging fibrosis or cirrhosis who achieved SVR[3]. Recent studies have reported that HCC occurrence and recurrence rates are potentially increased in HCV patients treated with DAAs[4-6]. This concern remains controversial due to the heterogeneous cohorts, variations in the inclusion criteria, and short duration of follow-up.

Persistent inflammation is a hallmark of chronic hepatic injury. HCV infection induces endogenous type I and III IFN activation, which activates natural killer (NK) cells[7] and leads to the expression of IFN-stimulated genes (ISGs)[8]. It causes profound alterations in the cytokine and chemokine signature in peripheral blood. HCV-specific CD8+ T cells play a central role in viral clearance. Chronic HCV infection is characterized by impaired HCV-specific CD8+ T cell responses resulting from viral escape and T cell exhaustion[9]. IFN-based therapy failed to recover the function of HCV-specific CD8+ T cells. This result suggested that the damage to CD8+ T cells might be permanent even after virus elimination[10]. In contrast, the combination of deleobuvir and faldaprevir resulted in the downregulation of programmed death-1, which led to rapid restoration of HCV-specific CD8+ T cell functions[11]. DAA-mediated HCV clearance is correlated with mitigation of the IFN-α-induced immune response, followed by the downregulation of CXCL10 and CXCL11 and normalization of the phenotype and function of NK cells[12].

It is unclear whether host immunological modification after viral eradication influences the development of HCC. Although DAAs are the first choice for HCV clearance, they are not sufficient to abolish hepatic inflammation. Long-term inflammatory responses may change the liver microenvironment and cause irreversible hepatocyte damage. A rapid decline in HCV viral load induced by DAAs results in the reconstitution of immune surveillance[4]. HCV eradication during DAA treatment is accompanied by downregulation of type II and III IFN, their receptors, and downstream ISGs[13], which may affect the antitumor activity of immune cells. IFNs have immunomodulatory properties that regulate various immune cells to inhibit tumor proliferation and angiogenesis. Unlike IFNs, DAAs have neither antiproliferative nor antiangiogenic properties, which may allow the proliferation of malignant cells.

The identification of HCV patients who maintain a high risk of HCC following successful antiviral therapy remains an unmet need. Hepatocarcinogenesis despite HCV clearance is still unclear. First, this study aimed to investigate the impact of differential cytokine expression profiles on the development of HCC among chronic hepatitis C patients with advanced fibrosis who achieved SVR. Second, we attempted to identify immune biomarkers to predict the risk of HCC after successful antiviral therapy.

MATERIALS AND METHODS

Subjects

One hundred treatment-naïve chronic hepatitis C patients with advanced fibrosis treated with either pegylated IFN/ribavirin or IFN-free DAA who achieved SVR were recruited from Kaohsiung Medical University Hospital since 2003. Patients were required to satisfy any one of the following criteria to be diagnosed with advanced fibrosis (F3/4): Fibrosis-4 (FIB-4) index > 3.25[14], transient elastography (Fibroscan) > 9.1 kPa, or acoustic radiation force impulse elastography > 1.81 m/s. The exclusion criteria were coinfection with hepatitis B, hepatitis D or human immunodeficiency virus; history of liver transplantation; prior presence of HCC; decompensated liver cirrhosis; malignancy; alcoholism; primary biliary cholangitis; α1-antitrypsin deficiency; autoimmune hepatitis; renal function impairment; and psychiatric conditions.

Treatment

In the IFN group, the patients were subcutaneously administered peginterferon α-2a (180 μg/wk) plus weight-based ribavirin (1200 mg/d for weights ≥ 75 kg or 1000 mg/d for weights < 75 kg) for 24 to 48 wk depending on the HCV genotype. In the

DAA group, the physician selected IFN-free DAA regimens for 12 to 24 wk that were discreetly based on the HCV international treatment guidelines (The Asian Pacific Association for the Study of the Liver, European Association for the Study of the Liver and American Association for The Study of Liver Diseases).

Outcome assessment

SVR was defined as undetectable HCV RNA throughout 12 wk (SVR12) for the DAA group or 24 wk (SVR24) for the IFN group after completion of antiviral therapy[15,16]. The primary endpoint was the occurrence of new-onset HCC. Standard HCC surveillance [abdominal ultrasound and α-fetoprotein (AFP) every six months] was performed during the follow-up[17]. HCC development within six months of initiation of antiviral treatment was excluded. Proof of HCC was directly linked to the National Cancer Registration of Taiwan in Health and Welfare Data Science Center (Taiwan). This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (No. KMUHIRB-E(I)-20180307 & KMUHIRB-G(II)-20170020). Written informed consent was acquired from all participants.

HCV genotyping and quantification

Anti-HCV antibodies were identified by a third-generation commercially available enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL, United States). HCV RNA was quantified by real-time polymerase chain reaction assay with a lower limit of detection of 12 IU/mL (RealTime HCV; Abbott Molecular, Des Plaines IL, United States)[18]. HCV genotypes were determined using a commercial kit (Abbott RealTime HCV Genotype II; Abbott Molecular, Des Plaines, IL, United States).

Cytokine measurement

Serum samples were collected from the participants at baseline and SVR24. In total, 64 serum cytokines and chemokines (Supplementary Tables 1-3) were measured by the magnetic bead multiplex immunoassay according to the manufacturer's instructions (Merck KGaA, Darmstadt, Germany)[19,20]. In brief, a calibration curve based on 1:3 dilutions of the highest standard was used for quantification. Beads were premixed and put into wells containing diluted serum and reagents. After fixation of the antigen on the capture antibody linked with the microspheres, a biotinylated detection antibody was added. The concentration of the analyte was quantified based on the bead color and the intensity of the fluorescent signal using the multiplex Luminex-200 (Luminex Corporation, Austin, TX, United States). All samples were analyzed in duplicate.

Statistical analysis

Student's *t* test and the Mann–Whitney *U* test were performed to compare the continuous variables. The chi-square (χ^2) test with Yates correction or Fisher's exact test was used to assess the categorical variables. Differences in the cumulative incidence of HCC between groups were analyzed by Kaplan-Meier survival analysis and the log-rank test. The risk factors for HCC were evaluated using multivariate Cox regression analysis. In conjunction with receiver operating characteristic area (ROC) analysis[21], the optimum cutoff value to distinguish between the risk strata was calculated by the Youden index[22]. The performance of biomarkers to predict the risk of HCC was calculated by time-dependent ROC curve analysis. The area under the ROC area (AUROC) was assessed by the timeROC package of R software (http://www.r-project.org). The statistical power for the comparison of survival curves between two groups under the Cox proportional hazards model was calculated by the powerSurvEpi package of R software. A two-tailed P value < 0.05 was considered statistically significant. The statistical analysis was conducted by the Statistic Packages for Social Science Program (SPSS v19.0 for Windows, SPSS Inc., United States). The statistical methods of this study were reviewed by Dr. Tsai PC from Kaohsiung Medical University.

RESULTS

Baseline demographics

The baseline demographics of the study subjects are shown in Table 1. There were no significant differences in age, sex, HCV genotype, FIB-4 index, or AFP levels between the DAA and IFN groups. HCV RNA was significantly higher in the DAA group than

Table 1 Baseline demographics of study subjects

Group	Total	DAA	IFN	*P* value (DAA *vs* IFN)
n	100	50	50	
Age (yr)	63.8 ± 7.2	64.9 ± 7.9	62.6 ± 6.3	0.100
Sex, *n* (%)				
Female	66 (66.0)	38 (76.0)	28 (56.0)	0.057
Male	34 (34.0)	12 (24.0)	22 (44.0)	
HCV genotype, *n* (%)				
1	65 (65.0)	37 (74.0)	28 (56.0)	0.098
2	23 (23.0)	10 (20.0)	13 (26.0)	
Mixed	12 (12.0)	3 (6.0)	9 (18.0)	
HCV RNA (log IU/mL)	2.47 ± 0.89	2.67 ± 0.84	2.28 ± 0.91	0.027
FIB-4	6.14 ± 3.28	6.55 ± 3.69	5.73 ± 2.80	0.213
AFP (ng/mL)	26.3 ± 56.7	28.8 ± 74.0	23.6 ± 29.3	0.662
Platelet (k/μL)	119.8 ± 34.7	115.2 ± 35.9	124.4 ± 33.2	0.186
AST (IU/L)	136.9 ± 79.9	115.2 ± 64.7	158.7 ± 87.9	0.006
ALT (IU/L)	177.5 ± 138.4	127.9 ± 83.9	227.0 ± 163.2	2.7×10^{-4}
γ-GT (IU/L)	67.6 ± 48.8	57.2 ± 42.6	76.7 ± 52.4	0.053
Cholesterol (mg/dL)	161.6 ± 34.8	158.6 ± 37.1	165.2 ± 32.0	0.388
Triglyceride (mg/dL)	97.7 ± 41.4	99.2 ± 40.5	96.0 ± 42.8	0.722
HDL (mg/dL)	47.0 ± 13.3	49.0 ± 11.6	44.1 ± 15.1	0.107
LDL (mg/dL)	90.6 ± 26.4	86.0 ± 24.4	97.2 ± 28.1	0.068
Cr (mg/dL)	0.79 ± 0.24	0.77 ± 0.27	0.82 ± 0.20	0.222
HbA1c (%)	5.8 ± 1.2	5.5 ± 0.7	6.0 ± 1.5	0.016
BMI (kg/m^2)	24.4 ± 4.7	24.0 ± 6.0	24.8 ± 2.9	0.381

DAA: Direct-acting antiviral agent; IFN: Interferon; HCV: Hepatitis C virus; FIB-4: Fibrosis-4 index; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: γ-glutamyltransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Cr: Creatinine; HbA1c: Hemoglobin A1c; BMI: Body mass index.

in the IFN group. Aspartate aminotransferase, alanine aminotransferase and hemoglobin A1c (HbA1c) were significantly elevated in the IFN group compared to the DAA group.

Cumulative probability of HCC development

HCC developed in 12 (IFN group *n* = 11, DAA group *n* = 1) of the 97 patients over 459 person-years of follow-up. Three patients were excluded because HCC occurred within six months of initiation of the antiviral treatment. The mean follow-up time was 7.46 years [interquartile range (IQR) = 3.65-12.23] in the IFN group and 1.84 years (IQR = 1.19-2.43) in the DAA group. The annual incidence of HCC was 2.95% in the IFN group and 1.16% in the DAA group. The Kaplan-Meier survival analysis showed no statistical significance in the accumulative probability of HCC between the IFN and DAA groups (log-rank *P* value = 0.712) (Figure 1).

Cytokines associated with HCC development

In total, 64 cytokines were used to analyze the relationship with HCC (Supplementary Table 1). Seven of the sixty-four cytokines were excluded from subsequent analysis because more than 80% of the samples were below the limit of detection. Members of the tumor necrosis factor (TNF) superfamily, including TNF-α and TNF-like weak inducer of apoptosis (TWEAK), were associated with the development of HCC. The baseline TNF-α level was significantly elevated in the HCC group compared

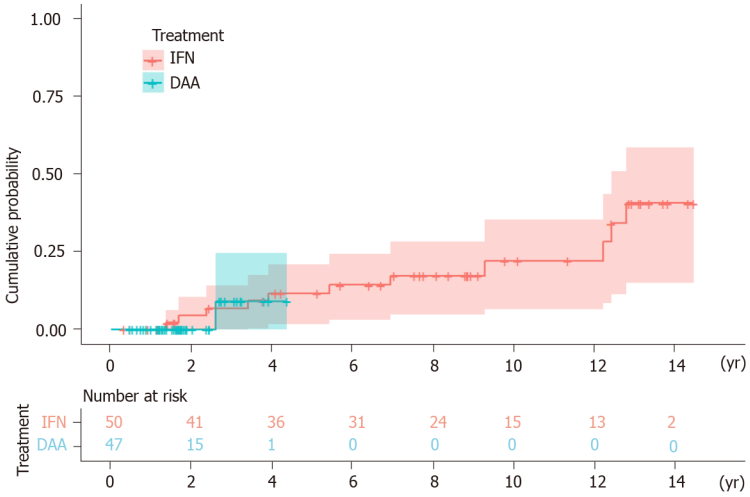


Figure 1 Kaplan-Meier survival analysis. HCC: Hepatocellular carcinoma; IFN: Interferon; DAA: Direct-acting antiviral agent.

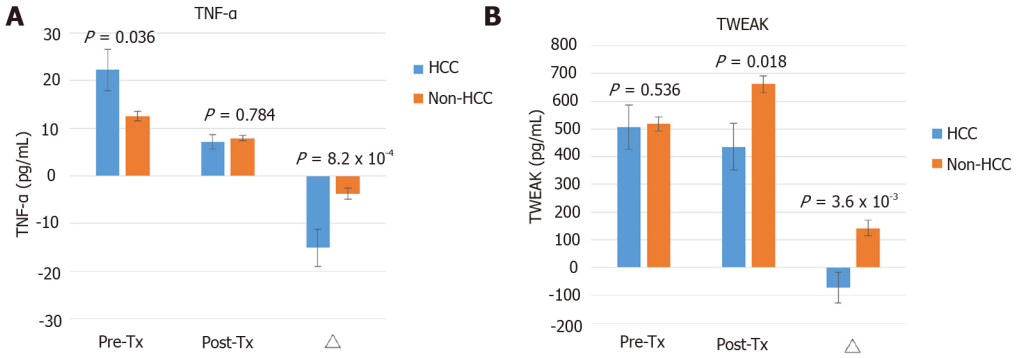


Figure 2 Cytokine expression between the hepatocellular carcinoma and non-hepatocellular carcinoma groups among chronic hepatitis C patients with advanced fibrosis. A: Tumor necrosis factor-α; B: Tumor necrosis factor-like weak inducer of apoptosis. Δ = (posttreatment cytokine level) - (pretreatment cytokine level). The bar represents the means ± SE. The *P* value was tested by the Mann–Whitney *U* test. HCC: Hepatocellular carcinoma; TNF-α: Tumor necrosis factor-α; TWEAK: TNF-like weak inducer of apoptosis; IFN: Interferon; Pre-Tx: Pretreatment; Post-Tx: Posttreatment.

to the non-HCC group (22.22 ± 4.33 *vs* 12.53 ± 1.03 pg/mL, $P = 0.036$). There was no significant difference in the posttreatment TNF-α levels between the HCC and non-HCC groups. The change in TNF-α levels (ΔTNF-α) before and after treatment significantly differed between the HCC and non-HCC groups (-15.11 ± 3.93 *vs* -3.78 ± 1.16 pg/mL, $P = 8.2 \times 10^{-4}$) (Figure 2A). The baseline TWEAK expression was similar in both groups. The posttreatment TWEAK level was significantly lower in the HCC group than in the non-HCC group (434.82 ± 84.18 *vs* 660.65 ± 30.34 pg/mL, $P = 0.018$). ΔTWEAK showed a reciprocal change between the HCC and non-HCC groups and achieved statistical significance (-71.78 ± 54.56 *vs* 142.81 ± 27.69 pg/mL, $P = 3.6 \times 10^{-3}$) (Figure 2B).

Among the HCV patients treated with pegIFN/ribavirin, the baseline TNF-α level was significantly higher in the HCC group than in the non-HCC group (22.55 ± 4.72 *vs* 9.13 ± 7.79 pg/mL, $P = 0.017$). The posttreatment TNF-α concentration was comparable between the HCC and non-HCC groups. ΔTNF-α levels significantly declined in HCC compared to non-HCC patients (-15.86 ± 4.22 *vs* -4.56 ± 1.85 pg/mL, $P = 0.007$) (Supplementary Figure 1A). The dynamic change in TWEAK did not show significant variations between the HCC and non-HCC groups (Supplementary Figure 1B).

Cox regression analysis of the relationship between the differentially expressed cytokines and HCC

In univariate Cox regression analysis, FIB-4 (≥ 9 *vs* < 9, crude HR = 4.04, 95%CI: 1.27-12.86, $P = 0.018$), HbA1c (≥ 7 *vs* < 7%, crude HR = 5.38, 95%CI: 1.38-20.99, $P = 0.015$), pretreatment TNF-α (≥ 18 *vs* < 18 pg/mL, crude HR = 5.15, 95%CI: 1.57-16.87, $P = 0.007$), ΔTNF-α (≤ -5.7 *vs* > -5.7 pg/mL, crude HR = 11.07, 95%CI: 2.27-53.87, $P = 0.003$), and ΔTWEAK (≤ -70 *vs* > -70 pg/mL, crude HR = 4.01, 95%CI: 1.20-13.40, $P = 0.024$)

were significant predictors of HCC. Multivariate stepwise Cox regression analysis revealed that ΔTNF-α was the only independent risk factor for HCC (HR = 11.54, 95%CI: 2.27-58.72, P = 0.003) (Table 2).

Among the HCV patients treated with pegIFN/ribavirin, univariate Cox regression showed that the significant predictors of HCC included sex, FIB-4 (≥ 9 *vs* < 9), HbA1c, baseline TNF-α (≥ 18 *vs* < 18 pg/mL) and ΔTNF-α (≥ -5.7 *vs* < -5.7 pg/mL). The association between ΔTWEAK (≤ -70 *vs* > -70 pg/mL) and HCC was borderline statistically significant. Stepwise multivariate Cox regression revealed that the ΔTNF-α level was the only independent risk factor for HCC in the IFN group (HR = 9.98, 95%CI: 1.88-52.87, P = 0.007 (Supplementary Table 2).

Subgroup analysis for the association between TNF-α and HCC

Since age and diabetes mellitus were important risk factors for HCC, the subjects were further stratified by age and HbA1c. The high- and low-risk groups were dichotomized based on ΔTNF-α with a cutoff value of -5.7 pg/mL. The multivariate Cox regression analysis revealed that the high-risk group (ΔTNF-α ≤ -5.7 pg/mL) had an 11-fold cumulative probability of HCC compared to that of the low-risk group (HR = 11.02, 95%CI: 1.86-65.17, P = 0.008) among HCV patients with HbA1c less than 7%. In the younger population (age < 65 years old), the HCC risk was borderline significant between the high- and low-risk groups (HR = 8.51, 95%CI: 0.78-92.86, P = 0.079). Among patients with both HbA1c < 7% and age below 65 years old, the high-risk group had a 20-fold cumulative probability of HCC in comparison with the low-risk group (HR = 19.99, 95%CI: 0.90-443.91, P = 0.058) (Figure 3). The level of ΔTNF-α did not influence the development of HCC in either the patients aged ≥ 65 years old or with HbA1c ≥ 7% (Supplementary Figure 2).

HCC predictive model

Based on previous analyses, the FIB-4 index, HbA1c, ΔTNF-α, and ΔTWEAK were selected as parameters to predict the risk of HCC. The HCC predictive model was as follows: Score = 4 × FIB-4 (≥ 9, yes = 1, no = 0) + 5 × HbA1c (≥ 7, yes = 1, no = 0) + 11 × ΔTNF (≤ -5.7, yes = 1, no = 0) + 4 × ΔTWEAK (≤ -70, yes = 1, no = 0).

The weighting coefficient for each parameter was derived from the crude hazard ratio of the univariate Cox proportional hazards model. The performance of this HCC predictive model was assessed by time-dependent ROC curve analysis. In overall cases, the 3-year, 5-year, 10-year, and 13-year areas under the ROC curve (AUCs) were 0.882, 0.864, 0.903, and 1.000, respectively (Figure 4A). In the IFN group, the 3-year, 5-year, 10-year, and 13-year areas under the ROC curve (AUCs) were 0.782, 0.802, 0.870, and 1.000, respectively (Figure 4B).

Kaplan-Meier analysis for HCV patients stratified by risk scores

To classify the predictive score according to the risk of HCC, the patients were further stratified into low- (score = 0-7), intermediate- (score = 8-15), and high-risk groups (score > 15). In the high-risk group, the 3-year, 5-year, and 10-year cumulative risks of HCC were 20.0%, 40.0%, and 60.0%, respectively. In the intermediate-risk group, the 3-year, 5-year, and 10-year cumulative probabilities of HCC were 11.4%, 16.9%, and 31.0%, respectively. In contrast, none of the low-risk patients had HCC within 14 years of follow-up after successful viral eradication among the overall cases (log-rank P value = 6.8×10^{-6}) (Figure 5A). Likewise, the Kaplan-Meier survival analysis revealed a significant difference in the cumulative probability of HCC among the IFN group stratified by the risk scores (log-rank P value = 9.6×10^{-5}) (Figure 5B).

DISCUSSION

This study revealed that there was no significant difference in the risk of HCC between the DAA and IFN groups after successful antiviral therapy. Downregulation of TNF-α and TWEAK increased the risk of hepatic carcinogenesis. ΔTNF-α was identified as an independent predictor of new-onset HCC among HCV patients with SVR. The effect of TNF-α was more prominent in young adults with normoglycemia. An HCC predictive model comprising FIB-4, HbA1c, ΔTNF-α, and ΔTWEAK had excellent performance, with 3-, 5-, 10-, and 13-year areas under the curve of 0.882, 0.864, 0.903, and 1.000, respectively. The 5-year accumulative risk of HCC was 0.0%, 16.9%, and 40.0% in the low-, intermediate-, and high-risk groups, respectively. These findings remained statistically significant among the HCV patients treated with pegIFN/ribavirin. Because there was only one HCC in the DAA group, the role of TNF-α in HCC should

Table 2 Factors associated with the onset of hepatocellular carcinoma: Univariate and multivariate Cox regression models

Variables	Univariate Cox regression		Multivariate Cox regression	
	Crude HR (95%CI)	*P* value	Adjusted HR (95%CI)	Adjusted *P* value
Age (yr)	1.07 (0.98-1.18)	0.149	-	-
Sex (male *vs* female)	3.19 (0.96-10.65)	0.059	-	-
HCV genotype	1.00 (0.47-2.13)	0.995	-	-
HCV RNA (log IU/mL)	0.68 (0.37-1.25)	0.213	-	-
FIB-4	1.13 (0.98-1.31)	0.089	-	-
FIB-4 (≥ 9 *vs* < 9)	4.04 (1.27-12.86)	0.018	-	-
Platelet (k/μL)	0.99 (0.98-1.01)	0.408	-	-
AFP (ng/mL)	1.00 (1.00-1.01)	0.308	-	-
HbA1c (%)	1.28 (1.01-1.62)	0.041	-	-
HbA1c (≥ 7 *vs* < 7%)	5.38 (1.38-20.99)	0.015	-	-
BMI (kg/m^2)	1.00 (0.83-1.20)	0.993	-	-
Treatment (DAA *vs* IFN)	0.66 (0.07-6.15)	0.713	-	-
TNF-α (pg/mL)				
Pre-Tx TNF-α ≥ 18	5.15 (1.57-16.87)	0.007	-	-
Post-Tx TNF-α ≥ 6	0.79 (0.25-2.46)	0.683	-	-
ΔTNF-α ≤ -5.7	11.07 (2.27-53.87)	0.003	11.54 (2.27-58.72)	0.003
TWEAK (pg/mL)				
Pre-Tx TWEAK ≥ 500	2.18 (0.64-7.39)	0.213	-	-
Post-Tx TWEAK ≥ 600	0.80 (0.20-3.11)	0.744	-	-
ΔTWEAK ≤ -70	4.01 (1.20-13.40)	0.024	-	-

The forward stepwise multivariate Cox regression model was adjusted by age, sex, hepatitis C virus (HCV) genotypes, HCV RNA, Fibrosis-4 index (FIB-4), platelet, alpha-fetoprotein, hemoglobin A1c, body mass index, treatment, tumor necrosis factor-α and tumor necrosis factor-like weak inducer of apoptosis (pretreatment, posttreatment, Δ). The cut-off value for each cytokine and FIB-4 was determined by Youden index of receiver operating characteristic curve. Δ = (posttreatment cytokine level) - (pretreatment cytokine level). DAA: Direct-acting antiviral agent; IFN: Interferon; HCV: Hepatitis C virus; FIB-4: Fibrosis-4 index; AFP: Alpha-fetoprotein; HbA1c: Hemoglobin A1c; BMI: Body mass index; HCV: Hepatitis C virus; TNF-α: tumor necrosis factor-α; TWEAK: TNF-like weak inducer of apoptosis; Pre-Tx: Pretreatment; Post-Tx: Posttreatment; HR: Hazard ratio; CI: Confidence interval.

be further verified in this population. The HCC risk could be modified by the pre-existing host background and adjusted by the immune signatures after viral eradication. This predictive model helps clinicians adopt appropriate surveillance strategies for chronic hepatitis C patients following successful antiviral therapy according to the risk of HCC.

Our study showed that elevation of pretreatment TNF-α levels raised the possibility of new-onset HCC. Consistent with our study, Tarhuni *et al*[23] found that HCV-related cirrhotic patients carrying TNF-α 308 G>A had higher basal TNF-α production and exhibited a higher risk of HCC. Elevated basal TNF-α indicates sustained hepatic inflammation accompanied by persistent liver damage, which is susceptible to carcinogenesis. A systematic review showed that TNF-α was one of the strongest host genetic predictors for HCC in HCV-infected patients[24]. These findings suggested that the immune background was affected before antiviral therapy.

Interestingly, the abrupt decline in TNF-α levels after successful antiviral therapy increased the risk of HCC in our study. This implies a potential modification of the immune milieu by antiviral therapy that may trigger HCC development. Stimulation of the immune system effectively protects tissues from malignant cell invasion. Both cytotoxic T lymphocytes and NK cells are potent effectors in immune surveillance. TNF-α mediates the immune response against tumor cells by creating a microenvironment toward immunogenic activation rather than suppression[25]. Suppression of TNF signaling enables tumor cells to evade attack by cytotoxic T lymphocytes and attenuate *in vivo* antitumor responses[26]. Antiviral therapy may disrupt the balance

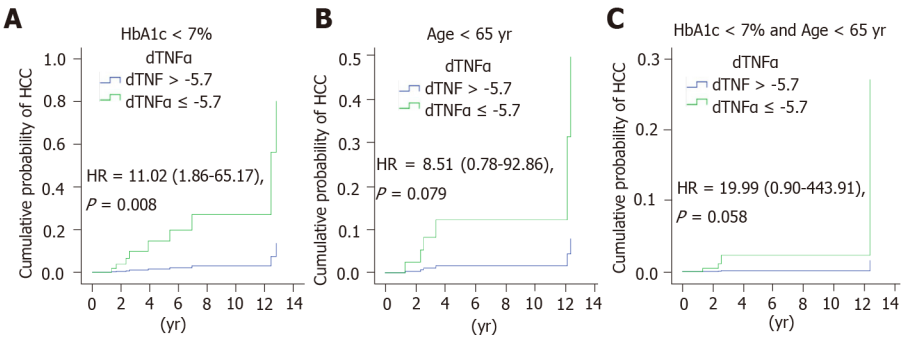


Figure 3 Multivariate Cox regression analysis of tumor necrosis factor-α associated with hepatocellular carcinoma in subgroups. Comparison of the cumulative probability of hepatocellular carcinoma development divided by Δtumor necrosis factor-α with a cutoff value of -5.7 pg/mL in patients with (A) hemoglobin A1c (HbA1c) < 7%, (B) age < 65 years old and (C) HbA1c < 7% and age < 65 years old. The *P* value was adjusted by age, sex, Fibrosis-4 index, and HbA1c. HR: Hazard ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; TNF-α: Tumor necrosis factor-α; HbA1c: Hemoglobin A1c.

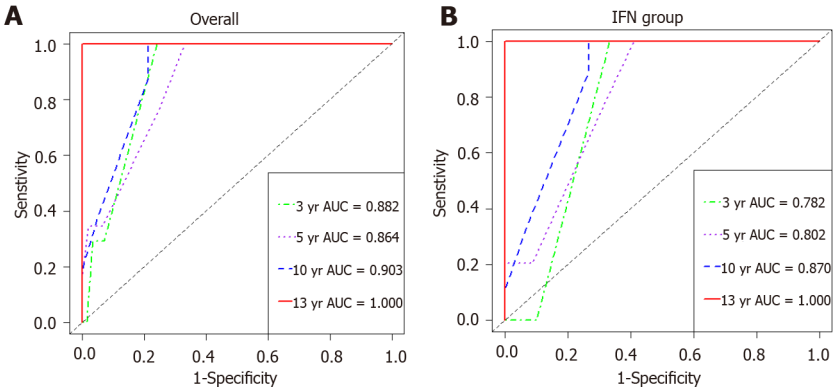


Figure 4 Time-dependent receiver operating characteristic curve analysis for the hepatocellular carcinoma predictive model. IFN: Interferon; AUC: Area under the curve.

from TNF-α activation to inhibition in immune surveillance. Alternatively, Debes *et al* [27] found that HCV patients with early-onset or recurrent HCC within 18 mo maintained stable or even higher TNF-α levels after DAA therapy. This implied that those patients might exhibit precarcinogenic or ongoing carcinogenic activity induced by TNF-α in response to occult HCC.

Both TNF-α and TWEAK belong to members of the TNF superfamily. These cytokines are mainly produced by macrophages, monocytes, and lymphocytes. TWEAK is a multifunctional cytokine that regulates a variety of cellular activities, including cell proliferation, differentiation, apoptosis, inflammation, and angiogenesis, *via* the fibroblast growth factor-inducible 14 receptor[28]. TWEAK appears to attenuate the innate response switch to adaptive immunity[29]. In addition, TWEAK is a weak inducer of apoptosis and also participates in tissue repair[30]. In chronic liver injury and repair, TWEAK appears to initiate liver progenitor cell expansion and ductal proliferation[31]. Hyperstimulation of inflammatory cells simultaneously results in excessive matrix deposition by activated hepatic stellate cells and myofibroblasts *via* the lymphotoxin-β signaling pathway[32]. Our study showed that posttreatment TWEAK expression was upregulated in the non-HCC group. Viral clearance alleviates the inflammatory status in the liver. It provides a microenvironment to facilitate the reconstruction of hepatocytes aided by TWEAK, which may further delay HCC development.

Our study confirms the consensus that DAA treatment does not markedly increase the risk of HCC compared to IFN treatment[33]. Most evidence has shown a decline in HCC risk regardless of whether SVR was achieved by IFN alone, DAA-only, or combined regimens[34]. However, successful antiviral therapy cannot eliminate the risk of HCC. The standard surveillance strategy (ultrasound and AFP every six months) advocates for all HCV patients. However, interindividual variations in HCC risk raise the question of whether the recommendations for HCC screening should be adjusted. Age, male sex, diabetes mellitus, and advanced fibrosis are well-known independent predictors of HCC after viral eradication[35-37]. In the absence of

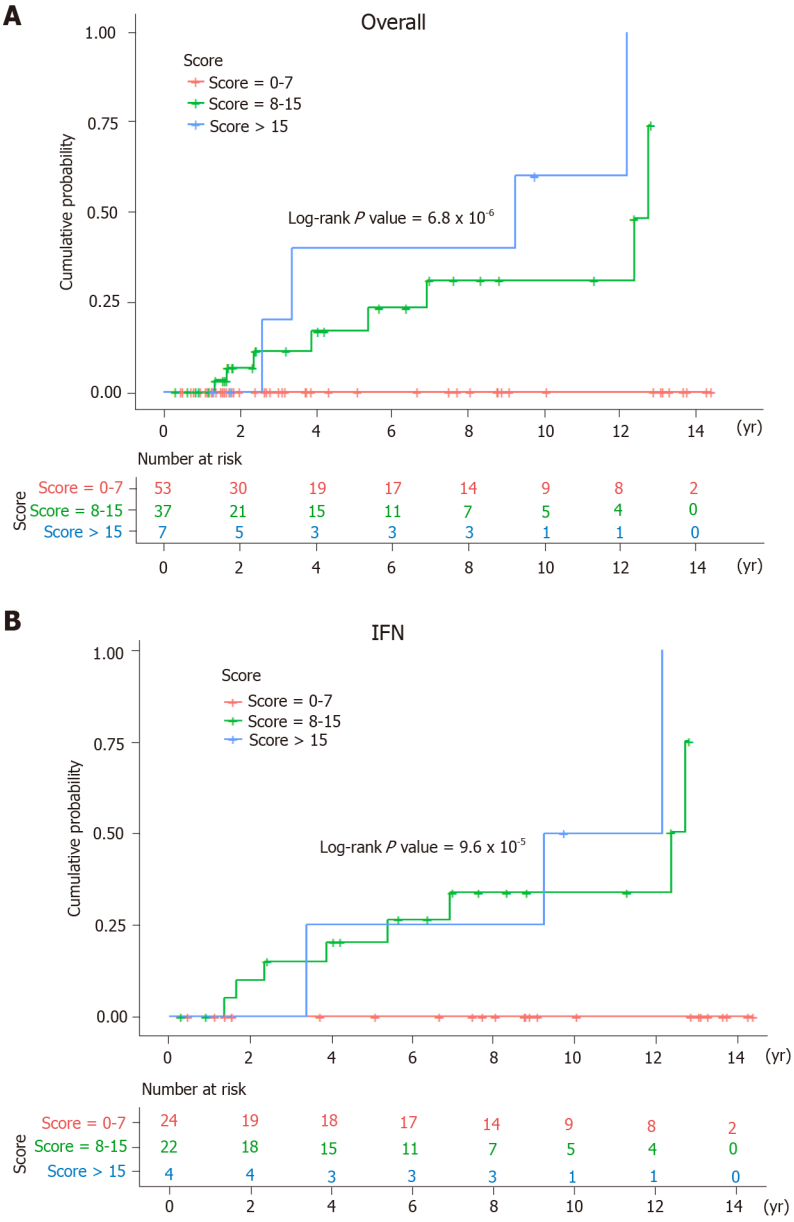


Figure 5 Kaplan-Meier survival analysis for chronic hepatitis C patients stratified by the risk scores. IFN: Interferon.

diabetes mellitus and old age (> 65 years old), the presence of ΔTNF-α ≤ -5.7 pg/mL increased the risk of HCC in patients with advanced fibrosis by 20-fold after HCV clearance. According to this HCC predictive model, patients with scores exceeding 15 should be closely monitored, since the 5-year cumulative risk of HCC reaches up to 40.0%. Nevertheless, none of the HCC cases had been identified over 14 years of follow-up in patients with a score of less than 7. The surveillance intervals may be extended among HCV patients achieving SVR in the absence of concurrent risk factors. In the post-DAA era, the risk model-based algorithm provides a cost-effective surveillance strategy for HCC.

The advancement of high-throughput technology makes early HCC detection more feasible. Currently, integrating multiomics data for HCC screening is also frequently observed[38]. The GALAD score consists of clinical factors (sex, age) and biomarkers (AFP, AFP-L3, and Des-carboxyprothrombin) that have an excellent performance to predict HCC, with an AUROC up to 0.97[39]. Using a miRNA panel (miR-22, miR-199a-3p) with AFP provided high diagnostic accuracy (AUROC = 0.988) for the early detection of HCC in HCV patients[39]. The methylation pattern of circulating cell-free DNA (APC, SFRP1, GSTP1, and RASSF1A) has demonstrated sufficient detection value to distinguish HCC patients from healthy controls[40]. Nevertheless, a majority of studies collected a cohort with a small sample size, and the analytical methods varied even in the same testing platform. These factors have limited the clinical application of these biomarkers.

Even though the sample size was limited in this pilot study, the statistical power was sufficient to be reliable. In overall cases, the statistical power of the association between ΔTNF-α and HCC was 99.9% to reject the null hypothesis at a *P* value < 0.05 under a hazard ratio of 11.54. In the IFN group, the statistical power of ΔTNF-α on HCC risk was 94.6% to reject the null hypothesis at a *P* value < 0.05 under a hazard ratio of 9.98. There are several limitations to this study. Although expensive IFN-free DAAs have been on the market since 2014, the National Health Insurance in Taiwan has reimbursed DAAs for HCV patients with advanced fibrosis since 2017. The follow-up time in most HCV patients treated with DAAs was less than 3 years. Owing to the small sample size and short follow-up time of the DAA group, a larger study cohort is necessary to validate the performance of this predictive model. IFN may induce distinct host immune alterations in comparison with DAA. As only one HCC case was identified in the DAA group throughout the follow-up period, it was unable to compare the diversity of cytokine profiles regarding HCC between the IFN and DAA groups. This predictive model was restricted to HCV patients with advanced fibrosis following successful antiviral therapy. Additionally, the optimal cutoff value should be further verified in other populations. The parameters of this predictive model were composed of serum cytokines involving the TNF superfamily. Host inflammation elicited by other etiologies may interfere with the predictive accuracy. Serum cytokines may not reflect the microenvironment within hepatocytes. To interpret this HCC predictive model, more care should be given to HCV patients presenting coinfection with other viruses, inflammatory disease, or malignancies.

CONCLUSION

This study revealed that downregulation of TNF-α increases the risk of HCC among HCV patients following successful antiviral therapy. Inhibition of TNF-α may attenuate host immune surveillance against tumor cells. Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification. With the development of high-throughput molecular technology, it is believed that more novel biomarkers will be applied in the early detection of HCC in the future.

ARTICLE HIGHLIGHTS

Research background

Successful hepatitis C virus (HCV) eradication cannot eliminate hepatocellular carcinoma (HCC) development. Chronic HCV infection induces profound alterations in cytokine and chemokine signatures. Clearance of HCV results in host immune modification, which may interfere with immune-mediated cancer surveillance.

Research motivation

The mechanism of hepatocarcinogenesis despite HCV clearance is still unclear.

Research objectives

To investigate the impact of differential cytokine expression on the development of HCC following HCV eradication.

Research methods

One hundred treatment-naïve HCV patients with advanced fibrosis who received antiviral therapy and achieved sustained virologic response (SVR) were enrolled. The primary endpoint was the development of new-onset HCC. In total, 64 serum cytokines were detected by the multiplex immunoassay at baseline and 24 wk after end-of-treatment.

Research results

The dynamics of serum tumor necrosis factor-α (TNF-α) and TNF-like weak inducer of apoptosis (TWEAK) were associated with HCC occurrence after HCV clearance. Multivariate Cox regression analysis showed that ΔTNF-α ≤ -5.7 pg/mL was an independent risk factor for HCC. An HCC predictive model comprising the Fibrosis-4 index, hemoglobin A1c, ΔTNF-α, and ΔTWEAK had excellent performance in stra-

tifying the risk of HCC among HCV patients with SVR.

Research conclusions

Downregulation of serum TNF-α significantly increased the risk of HCC after HCV eradication.

Research perspectives

Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification.

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SYSTEMATIC REVIEWS

Current guidelines for the management of celiac disease: A systematic review with comparative analysis

Alberto Raiteri, Alessandro Granito, Alice Giamperoli, Teresa Catenaro, Giulia Negrini, Francesco Tovoli

ORCID number: Alberto Raiteri 0000-0003-3051-8487; Alessandro Granito 0000-0002-0637-739X; Alice Giamperoli 0000-0003-3717-8104; Teresa Catenaro 0000-0001-5333-8089; Giulia Negrini 0000-0002-9261-2316; Francesco Tovoli 0000-0002-8350-1155.

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Alberto Raiteri, Alessandro Granito, Alice Giamperoli, Teresa Catenaro, Giulia Negrini, Francesco Tovoli, Division of Internal Medicine, Hepatobiliary and Immunoallergic Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna 40138, Italy

Corresponding author: Francesco Tovoli, MD, Assistant Professor, Research Fellow, Division of Internal Medicine, Hepatobiliary and Immunoallergic Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna, *via* Albertoni 15, Bologna 40138, Italy. francesco.tovoli2@unibo.it

Abstract

BACKGROUND

Wheat and other gluten-containing grains are widely consumed, providing approximately 50% of the caloric intake in both industrialised and developing countries. The widespread diffusion of gluten-containing diets has rapidly led to a sharp increase in celiac disease prevalence. This condition was thought to be very rare outside Europe and relatively ignored by health professionals and the global media. However, in recent years, the discovery of important diagnostic and pathogenic milestones has led to the emergence of celiac disease (CD) from obscurity to global prominence. These modifications have prompted experts worldwide to identify effective strategies for the diagnosis and follow-up of CD. Different scientific societies, mainly from Europe and America, have proposed guidelines based on CD's most recent evidence.

AIM

To identify the most recent scientific guidelines on CD, aiming to find and critically analyse the main differences.

METHODS

We performed a database search on PubMed selecting papers published between January 2010 and January 2021 in the English language. PubMed was lastly accessed on 1 March 2021.

RESULTS

We distinguished guidelines from 7 different scientific societies whose reputation is worldwide recognized and representative of the clinical practice in different geographical regions. Differences were noted in the possibility of a no-biopsy diagnosis, HLA testing, follow-up protocols, and procedures.

CONCLUSION

Grade A (Excellent): 0
Grade B (Very good): B, B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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We found a relatively high concordance between the guidelines for CD. Important modifications have occurred in the last years, especially about the possibility of a no-biopsy diagnosis in children. Other modifications are expected in the next future and will probably involve the extension of the non-invasive diagnosis to the adult population and the follow-up modalities.

Key Words: Celiac disease; Gluten; Gluten-free diet; Gluten sensitivity; Clinical guidelines; Non-invasive diagnosis; Histopathological findings; Serological markers; Genetics

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Core Tip: Once considered a rare condition, celiac disease (CD) is becoming a significant health issue globally. An increasing number of studies have investigated this condition. International scientific societies have proposed guidelines for the management of CD to translate this evidence into clinical practice. In this review, we critically analyse both the converging and diverging points in the current clinical guidelines of CD, focusing on the diagnostic aspects and follow-up procedures.

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INTRODUCTION

Celiac disease (CD) is an immune-mediated reaction to gluten characterised by an inflammatory injury to the small bowel in genetically predisposed subjects as a result of an inappropriate T cell-mediated immune response[1].The epidemiology of CD is well known, with an estimated worldwide prevalence of 0.6%-1% of the general population[2]. However, CD remains largely underdiagnosed in developing countries and has a higher impact on children[3,4]. Simultaneously, the misdiagnosis of CD is becoming an emergent problem worldwide[5].

An evidence-based approach is needed to optimise diagnostic accuracy to avoid life-threatening complications (including small bowel carcinoma and lymphoma)[6] resulting from unrecognised CD on the one hand, and unnecessary cost burden and impact on the quality of life due to incorrect prescription of a life-long gluten-free diet (GFD) on the other hand.

Simultaneously, follow-up of patients with CD who are on a GFD is of critical importance to assess the responsiveness to the GFD, detect complicated CD, find associated autoimmune diseases, and identify metabolic alterations induced by the GFD[7].

Thus, an increasing number of scientific societies have proposed guidelines for diagnosing and managing CD. In our systematic review, we identified the most recent and significant national and international guidelines and compared their recommendations. We also underlined the most apparent differences among these guidelines to identify 'hot topics' on CD and possible future developments.

MATERIALS AND METHODS

The primary aim of this review was to identify the most recent national and international guidelines for CD by means of a systematic review and to compare their main recommendations.

We performed a database search on PubMed and selected papers published between January 2010 and January 2021 in the English language. PubMed was last accessed on 1 March 2021. The following keywords and terms were used: (1) *Coeliac Diseaseor Celiac Disease*; (2) *Guideline*; and (3) *Management*. The following string was used: (("coeliac disease"[All Fields] OR "celiac disease"[MeSH Terms] OR ("celiac"[All

Fields] AND "disease"[All Fields]) OR "celiac disease"[All Fields] OR ("coeliac disease"[All Fields] OR "celiac disease"[MeSH Terms] OR ("celiac"[All Fields] AND "disease"[All Fields]) OR "celiac disease"[All Fields])) AND ("guideline"[Publication Type] OR "guidelines as topic"[MeSH Terms] OR "guideline"[All Fields] OR ("manage"[All Fields] OR "managed"[All Fields] OR "managements"[All Fields] OR "managements"[All Fields] OR "manager"[All Fields] OR "manager s"[All Fields] OR "managers"[All Fields] OR "manages"[All Fields] OR "managing"[All Fields] OR "management"[All Fields] OR "organization and administration"[MeSH Terms] OR ("organization"[All Fields] AND "administration"[All Fields]) OR "organization and administration"[All Fields] OR "management"[All Fields] OR "disease management"[MeSH Terms] OR ("disease"[All Fields] AND "management"[All Fields]) OR "disease management"[All Fields]))).

A total of 415 papers were identified with no duplicates, and, as a first step, no papers were excluded for other reasons (PRISMA flow diagram reported in Figure 1). However, twenty-one records were unavailable, leaving 396 papers for further evaluation. As a second step, we excluded papers that were not pertinent to any of the following criteria: (1) Clinical guidelines related to diagnosis and management of CD; and (2) Clinical guidelines published by governmental agencies and scientific associations. We included only the last version of the guidelines, excluding the previous updated versions.

According to the selection criteria, out of the 396 results of PubMed research assessed for eligibility, seven guidelines were finally included in this analysis. These guidelines strictly focus on the diagnosis and management of CD. These papers are presented in order of publication (newest to oldest): (1) European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020[8]; (2) European Society for the Study of Coeliac Disease (ECD) 2019[9]; (3) World Gastroenterology Organization (WGO) 2017[10]; (4) Central Research Institute of Gastroenterology, Russia, 2016[11]; (5) National Institute for Health and Care Excellence (NICE), 2015 [12]; (6) British Society of Gastroenterology (BSG), 2014[13]; and (7) America College of Gastroenterology (ACG), 2013[14].

The recommendations provided by each selected guideline were systematically explored and classified into five categories: patients to be tested for CD, diagnostic tests (serology, duodenal biopsy, genetic test, no-biopsy diagnosis), potential/silent/seronegative CD, refractory/complicated CD, and follow-up. These categories represent the most discussed topics of CD.

The results are reported in different paragraphs, containing both a brief introduction to the specific topic (with references derived from the supporting evidence used by the guidelines and other relevant papers according to a narrative approach) and a comparative analysis of the guidelines' recommendations (collected using a strictly systematic approach).

RESULTS

Clinical presentation and risk factors: who should be tested for CD?

CD is a diagnostic challenge as it may develop at any age (even in older adults) and with a polymorphic clinical presentation[15]. The clinical spectrum of CD includes both symptomatic and silent forms revealed only by serological screening[16,17]. CD-related symptoms can be both intestinal and extraintestinal, reflecting the systemic nature of the disease. These manifestations are classified as 'classical' and 'non-classical' according to the historical presentation of first described cases. Table 1 reports the main manifestations of CD according to their categorization[1,17-26].

Some guidelines draw specific attention to some extraintestinal symptoms (Figure 2). In particular, the ESsCD 2019 guidelines focus on oral-dental and neuropsychiatric manifestations[9]. CD testing is advised in cases of dental enamel defects and recurrent oral aphthae. Special attention to neurological manifestations has also been drawn by the Russian Central Research Institute of Gastroenterology[11]. These guidelines also focus on reproductive disorders, such as delayed sexual development, amenorrhea, infertility, and miscarriage[11].

Despite these premises, all the guidelines agree on testing for CD in children, adolescents, and adults showing classical and non-classical symptoms of CD[7-13]. There is also a consensus on considering iron-deficiency anaemia and hypertransaminasemia as the most common laboratory abnormalities[8-14].

Table 1 Most frequent clinical manifestions of celiac disease

	Intestinal	Extraintestinal
Classical	Diarroea	Iron deficiency anaemia
	Failure to thrive	Muscle waisting
	Weight loss	Oedema
	Bloating	
Non classical	Chronic abdominal pain	Short stature
	Abdominal distension	Delayed puberty
	Constipation	Amenorrhea
	Vomiting	Irritability, unhappiness
		Chronic fatigue
		Epilepsy
		Peripheral neuropathy
		Joint/muscle pain
		Elevated aminotransferases
		Aphtous stomatitis
		Recurrent miscarriages
		Reduced bone mineral density

The high-risk group of patients did not change over time. These groups include first-degree relatives of patients with CD, patients with autoimmune conditions (such as type 1 diabetes mellitus and thyroid diseases) or genetic disorders such as IgA deficiency, Down syndrome, Turner syndrome, and Williams-Beuren syndrome[8-14].

Diagnosis.

There is no 'gold standard' for the diagnosis of CD. Clinical features, serology, or histology alone cannot provide a definitive diagnosis. Instead, the final diagnosis of CD relies on a combination of these elements. All the guidelines agree on a sequential approach to diagnosis, consisting of serology as a first-line test in high-risk patients, followed by duodenal biopsy in cases of positive serology or persistent suspicion of malabsorption (Figure 3). A positive serology paired with evidence of duodenal villous atrophy indicate a definite CD diagnosis, whereas cases with discordant findings should undergo HLA testing. All the guidelines also agree that patients with dis-cordance between serology, histology, and HLA DQ2/DQ8 positivity should be evaluated on a patient-by-patient basis in expert centres. The so-called 'four-out-of-five rule' has long been advocated as a standard of care[27]. According to this rule, four of the following criteria are sufficient to establish CD diagnosis: (1) Typical signs and symptoms (diarrhoea and malabsorption), (2) Antibody positivity, (3) HLA-DQ2 or HLA-DQ8 positivity, (4) Intestinal damage (*i.e.*, villous atrophy and minor lesions); and (5) Clinical response to GFD. This rule also helps physicians to identify various subtypes of CD, that is, non-classic CD (absence of point 1), seronegative CD (absence of point 2), potential CD (absence of point 4), and non-responsive CD (absence of point 5). However, the 'four-out-of-five rule' is yet to be recognised by any guideline.

We will report the guidelines' detailed suggestions for obtaining key diagnostic elements from serology, histology, and genetic testing in the following paragraphs.

Serology

All diagnostic serological testing should be performed in patients on a gluten-containing diet[28]. Serum immunoglobulin A(IgA) anti-tissue transglutaminase antibody (anti-tTG-IgA) is widely accepted as the most sensitive test for CD diagnosis, although it suffers from low specificity, especially at low titres[29-33]. In contrast, IgA anti-endomysial antibodies (EMA-IgA) are nearly 100% specific for CD but are less sensitive, more expensive, and more operator-dependent than anti-tTG-IgA. Therefore, these characteristics make EMA-IgA an ideal second-line test[34]. The diagnostic performance of both anti-tTG-IgA and EMA-IgA is limited in patients with concurrent

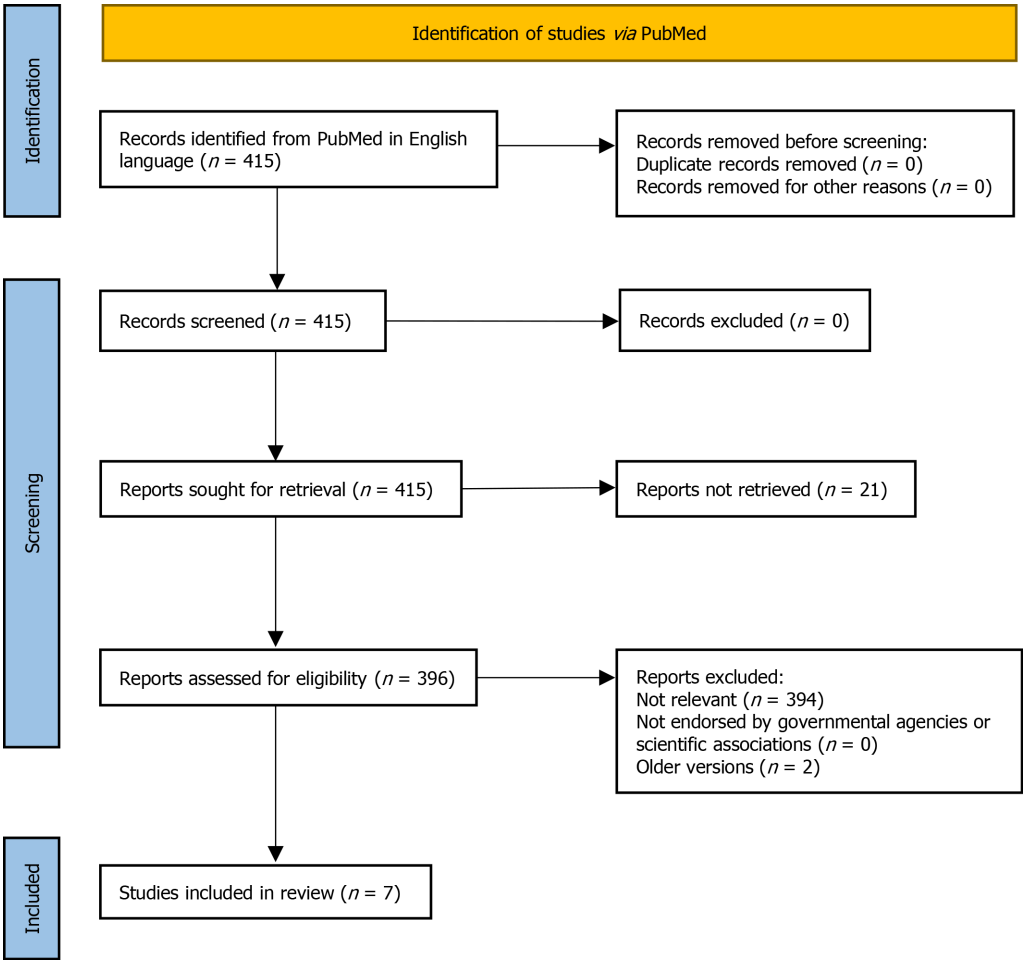


Figure 1 PRISMA flow diagram.

IgA deficiency. Antibodies to deamidated gliadin peptides (DGP) of the IgG class are advantageous in this setting and for younger children[35,36]. Even with the most recent advancements in CD serology, up to 2% of patients with CD have no circulating markers of gluten sensitivity, defining a condition of seronegative CD[37].

Currently, the guidelines are concordant and suggest anti-tTG-IgA as the initial serological test, complemented by a determination of total IgA levels to rule out concurrent IgA deficiency (Figure 4)[8-14]. This initial approach was suggested for both children and adults.TheACG2013 guidelines suggest a combination of different IgA and IgG antibodies in children younger than two years of age (for instance, anti-tTG IgA and DGP-IgG)[14]. This approach is still accepted only by the WGO2017 guidelines[10]. The remaining guidelines advise against this strategy, as a combination of antibodies implies a higher sensitivity at the expense of a reduced specificity, often leading to the necessity of histological confirmation. This scenario represents an obstacle in the pursuit of a no-biopsy approach in children, for whom the anti-tTG-IgA + total IgA strategy fits better[8]. Alternatively, DGP-IgG (together with anti-tTG-IgG) maintained the unanimous recommendation as the test of choice in patients with IgA deficiency[8-14].

Further, EMA-IgA is considered a confirmatory test, particularly when TG2 has a low titre, *i.e.*,< 2x the upper normal limit (UNL)[9,10,12]. A positive result is also required for a no-biopsy CD diagnosis in children with anti-tTG IgA > 10x[8]. However, the use of paired anti-tTG and EMA-IgA as the first diagnostic test is not supported by any guideline.

Currently, all of the guidelines strongly discourage urine, stool, and saliva tests in clinical practice due to their low-performances[8-14] and the consequent risk of initiating a GFD without a firm diagnosis, impacting the final diagnosis[13].

Biopsy

For a long time considered the 'gold standard' for diagnosing CD (ambiguously suggesting that other tests were of lesser importance), duodenal biopsies remain the

Recommendations		Change over time
In children, classical symptoms of malabsorption are more specific. Some non-classical symptoms are more specific than others (including iron deficiency anaemia, diarrhoea IBS-like, chronic constipation, and enamel defects)		No major changes over time
In adults, sensitivity and specificity of classical and non-classical symptoms are moderate. Testing for CD among individuals with only subtle and non-classical symptoms is advised		No major changes over time
Consider testing for CD in high-risk groups such as CD first-degree relatives, patients with autoimmune conditions such as type 1 Diabetes Mellitus, thyroid disease, liver disease, patients with genetic conditions such as Down syndrome, Turner syndrome, Williams-Beuren syndrome and IgA deficiency		No major changes over time

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

Central Research Institute of Gastroenterology, Russia, 2016 (11).

National Institute for Health and Care Excellence (NICE), 2015 (12).

British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 2 Recommendations about case finding.

mainstay of CD diagnosis, and all guidelines unanimously recognise this role. The presence of positive histology, however, was not considered CD-specific. Thus, clinical, and serological correlations are mandatory (Figure 5) [8-14].

Duodenal biopsies should be obtained from all patients with suspected CD. In high-risk symptomatic patients, duodenal biopsies should be performed irrespective of serology results for CD[9,13,14]. Some authors also suggested that duodenal biopsies should be considered in any individual undergoing endoscopy because of the relatively high prevalence of CD in the general population and its polymorphic presentation[13].

Histology samples should be collected from multiple sites, given the possible patchy distribution of CD lesions. Current evidence suggests collecting four biopsies from the second duodenal portion and two biopsies from the bulb[38]. Biopsy sample orientation using cellulose acetate Millipore filters is of paramount importance to avoid artefacts, potentially leading to a false diagnosis of villous atrophy[39].

The histological findings are currently categorised according to the classification proposed by Marsh and subsequently modified by Oberhuber[40]. Pathology findings are reported as Marsh-Oberhuber 0 (normal histology), 1, 2, or 3 (subdivided into 3a, 3b, and 3c).

An increase in intraepithelial lymphocytes (IELs) without villous atrophy defines Marsh 1 Lesion. In most cases, Marsh 1 Lesions (also called minimal lesions) are attributable to other causes, including lymphocytic colitis, bacterial and parasitic intestinal infections (especially *Helicobacter pylori* and *Giardia lamblia*), small intestinal bacterial overgrowth, Crohn's disease, common variable immunodeficiency, and non-steroidal anti-inflammatory drugs[41]. While a Marsh 1 Lesion is not considered sufficient to diagnose CD, the BSG 2014 guidelines state that minimal lesions combined with positive serology could represent a probable CD. A trial with a GFD could be considered to support the diagnosis of CD[13]. When the increase in IELs is paired with hyperplasia of the duodenal crypts, the lesion is classified as Marsh 2.

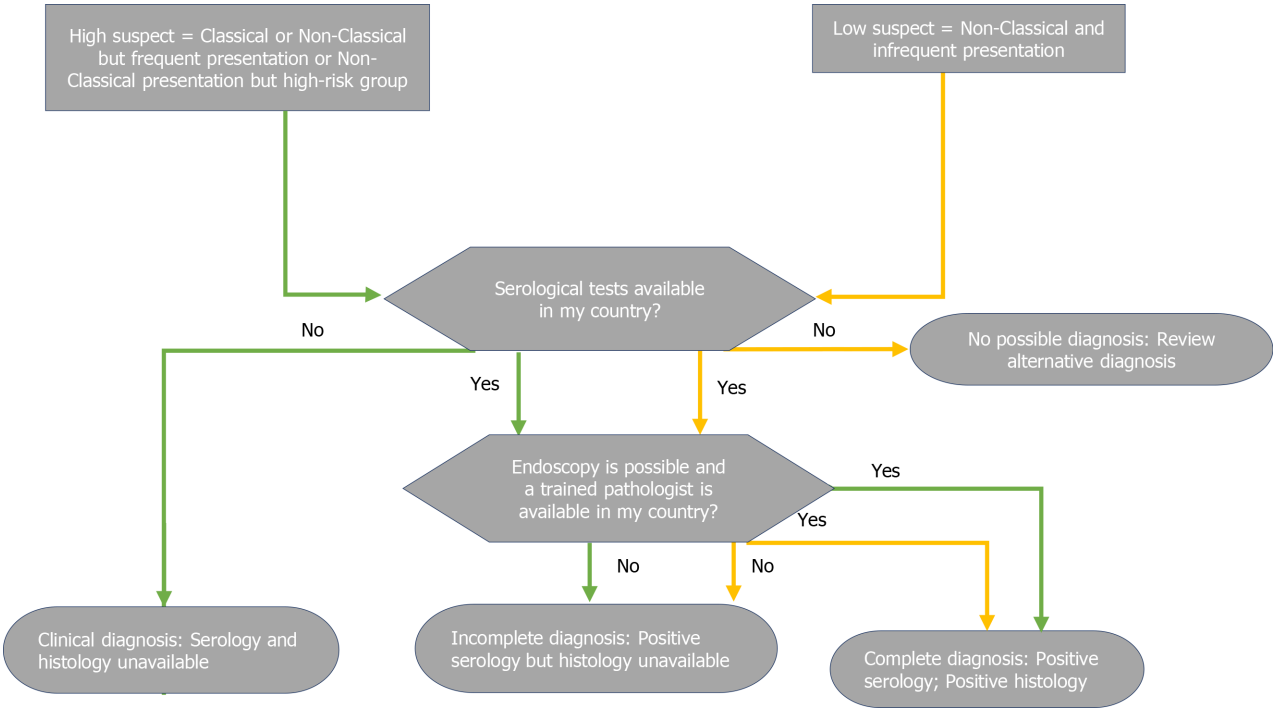


Figure 3 Worldwide adapted decision-making process for diagnosing celiac disease. Highly suspicious celiac disease (CD) comprises "classical presentation" (*i.e.*, classical symptoms in children include failure to thrive, weight loss, growth failure, vomiting, chronic diarrhea, bloating, Iron-deficiency anemia, muscle wasting, oedema due to hypoproteinemia, irritability and unhappiness; in adults, classical symptoms include chronic diarrhea, weight loss, iron-deficiency anemia, malaise and fatigue, oedema due to hypoproteinemia, and osteoporosis), frequent "non-classical presentation" (*i.e.*, iron deficiency and hypertransaminasemia) and "non-classical presentation" but high risk group (*i.e.*, CD first-degree relatives, autoimmune conditions such as type 1 Diabetes Mellitus, and thyroid disease, genetic conditions such as IgA deficiency, Down syndrome, Turner syndrome and Williams-Beuren syndrome).

Conversely, increased IELs in combination with villous atrophy define the typical CD lesion (Marsh 3), subclassified as mild (3a), moderate (3b), or subtotal (3c)[40]. Some authors proposed a simplified histopathological grading, reducing the possible grades from five to three, thus reducing the possible inter-operator variability in the histological interpretation[42].This simplified classification is yet to be adopted by the international guidelines, which currently recommend the Marsh-Oberhuber classification[8-14].

At present, there is no alternative to duodenal biopsy for examining mucosal damage[8-14]. For instance, in children, video-capsule endoscopy (VCE) gives no indications[8], although in adults, VCE could support the diagnosis in cases of discordance between serology and biopsy[13] or if the patient is unwilling or unable to undergo traditional endoscopy[14]. VCE could also play a role in detecting CD complications (*i.e.*, lymphoma, adenocarcinoma, ulcerative jejunitis)[9] and in helping to differentiate extended diseases (*e.g.*, CD *vs* proximal Crohn's disease)[11]. Anti-actin IgA antibodies have been shown to be predictive of severe villous atrophy in CD patients at the time of diagnosis[43]. Theoretically, they may also provide indirect information about villous recovery following the introduction of the GFD; however, data are still lacking in this setting. The available information about faecal and salivary microbiome, at present, is not sufficient to allow a reliable conclusion for the diagnosis of CD[44,45]. Intestinal fatty-acid binding protein (I-FABP) are higher in dietary non-adherence and unintentional gluten intake and could be used as a sensible blood marker of mucosal damage[46,47].This exam was first mentioned in the ESsCD guidelines[9].

A repeated small intestinal biopsy, including biopsies from the jejunum, could be considered in adults with discordance between histopathology and anti-tTG-IgA results[13]. In children, re-cutting biopsies and/or a second opinion from an experienced pathologist is preferred over endoscopic repetition[8].

In adults, a gluten challenge should be proposed for patients with uncertain CD diagnosis, who have been started on a GFD[9-14]. In children, gluten challenge is discouraged before the age of 5 years and during puberty, and in general, it should be reserved for unusual cases[8].

Recommendation		Change over time
Anti-tissue Transglutaminase 2 IgA (TGA-IgA) should be used as the initial serological test, complemented by total IgA value in children of any age and adults		Changes over time
In patients with low total IgA concentrations, an IgG-based test, preferably TGA-IgG or DGP-IgG, should be performed as a second step		No major changes over time
A strategy based on a combination of antibodies addressing the same target (*i.e.*, TGA-IgA and EMA-IgA) as a first approach is not recommended		No major changes over time

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National Institute for Health and Care Excellence (NICE), 2015 (12).

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Figure 4 Recommendations about serology. IgA: Immunoglobulin A; IgG: Immunoglobulin G; DGP: Deamidated gliadin peptides; EMA: Anti-endomysium antibodies.

Gluten challenge protocols are not homogeneous. A diet containing at least 10 g of gluten per day for 6-8 wk seems to be the most effective way to achieve disease relapse; however, the evidence is weak[28]. In shorter protocols, a diet containing at least 3 g of gluten per day for at least 2 wk seems to be sufficient for most patients[10,13,14]. Certainly, a shorter and lighter approach would fit better for highly symptomatic patients. A strategy for optimising the result would be to undergo a serology test after two weeks and, if negative, to extend the challenge to 8 wk[13].

After reintroducing gluten, physical symptoms should not be used for diagnosis in the absence of other supportive evidence[8,9,11-14]. A diagnosis based only on the disappearance of symptoms on GFD and relapse during gluten re-introduction can be relevant in geographic areas where serology tests are not available, as the only way to confirm the diagnosis and treat the disease[10].

Human Leukocyte Antigen testing

The strong genetic component of CD is testified by its high familial recurrence and high disease concordance among monozygotic twins (75%-80%)[48]. The presence of human leukocyte antigen (HLA) -DQ2/DQ8 is a pathogenic requisite for the development of the typical immune alterations found in CD. Simultaneously, HLA DQ2/DQ8 can be found in up to 30%-40% of the general population, so its specificity is remarkably poor[49]. In contrast, the absence of HLA DQ2/DQ8 virtually excludes CD diagnosis[48,49].Restricting this observation to the sole HLA DQ2 alleles, a recent systematic review of the literature confirmed that only 5.06% of patients with CD were completely lacking the HLA-DQB1*02 allelic variant[50].

Consequently, all the guidelines advise against using HLA testing as a first-line tool for the diagnosis of CD (Figure 6)[8-14]. They are also concordant in allocating this resource for: (1) Patients with uncertain diagnosis of CD, already on a GFD; (2) Patients with a flat intestinal mucosa but negative serology; and (3) In patients already on a GFD, serology and histology can be inconclusive. In this context, before embarking on a so-called 'gluten-challenge', it is advisable to verify the presence of HLA-DQ2/DQ8[8-14].

Recommendation		Changes over time
Adult patients with a positive serology must undergo endoscopy with duodenal biopsies to achieve a final diagnosis		With exceptions
In children, in precise conditions, diagnosis can be achieved without a duodenal biopsy		Major changes over time
Duodenal biopsy should be performed, irrespective of positive serology for CD, in case of high clinical suspicion of CD		No major changes over time
At least 4 biopsies from the distal duodenum and at least 1 from the duodenal bulb should be taken for histology assessment during a gluten-containing diet		No major changes over time
The diagnosis is confirmed in the presence of Marsh ≥ 2 lesions. Marsh 1 is not sufficient to diagnose CD		No major changes over time. Minor exceptions
A gluten challenge should be proposed to patients who have been started on a GFD but have a doubtful diagnosis		Minor changes over time and guidelines
A diagnosis based only on the disappearance of symptoms on GFD and relapse during gluten re-introduction is absolutely discouraged.		With exceptions
No exams can surrogate mucosal damage without biopsy		No major changes over time. Minor exceptions

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Figure 5 Recommendations about serology.

HLA tests would be useless for patients with positive serology before a gluten-challenge because virtually 100% of those patients would be positive. Therefore, HLA typing is no longer a criterion for the 'no-biopsy' approach of diagnosis in children with a TGA-IgA > 10x UNL[8]. In patients with positive histology (*i.e.,* villous atrophy, though occasionally detected on esophagogastroduodenoscopy), and negative or questionable serology, HLA testing can exclude the diagnosis of CD[9]. In contrast, a positive result cannot confirm the diagnosis, which should be carefully evaluated on a patient-by-patient basis in expert centres.

The use of HLA typing in high-risk populations is controversial. HLA-DQ2/DQ8 can be found in more than 50% of first-degree relatives of patients with CD and in patients with other autoimmune or genetic disorders related to CD[14,49]. Most

Recommendation		Changes over time
HLA -DQ2/DQ8 testing has only a high negative predicting value and is recommended in selected patients to rule out coeliac disease in patients: Already on a gluten-free diet With a negative or questionable serology but positive histology		No major changes over time
HLA -DQ2/DQ8 testing is not recommended alone or combined with serology tests to confirm the diagnosis		No major changes over time
HLA) -DQ2/DQ8 testing in high-risk populations can indefinitely exclude these patients from a periodic screening		Minor changes over time

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Figure 6 Recommendations about Human Leukocyte Antigen testing.

guidelines suggest excluding HLA-DQ2/DQ8 in CD first-degree relatives and high-risk patients, even if asymptomatic, to avoid periodic monitoring[9,10,13,14]. This strategy can be questioned in terms of resources and costs[10,11,14]. Some authors hav esuggested screening high-risk patients only if they complain of gastrointestinal or extraintestinal symptoms or have laboratory abnormalities[11]. In addition, a two-step genetic screening procedure starting with HLA-DQ β chains has been proposed[51]. Thus, the choice of screening for symptomatic or asymptomatic first-degree relatives or high-risk patients, with or without a preliminary determination of HLA-type, remains debated, needing to take local resources and cost-benefit rates into account.

No-biopsy diagnosis

While most guidelines allow a no-biopsy diagnosis in children under strict conditions, endoscopy with duodenal biopsies is still mandatory to achieve a final diagnosis of CD in adults[9-14]. As the only exception, the WGO guidelines allow a diagnosis based on serology and clinical response to the GFD (Figure 7) in developing countries where endoscopy may not possible or trained pathologists may not be available[10].

The ESPGHAN2012 guidelines endorsed the possibility of a no-biopsy approach in children for the first time. This possibility was limited to certain conditions, which included the presence of classic symptoms, with tTG-IgA > 10x UNL, EMA-IgA positivity, and presence of permissive HLA[8].

This approach was subsequently adopted by a plurality of international guidelines [9-12]. although, the ACG2013 and BSG 2014 guidelines did not include this approach [13,14].

The 2020 update of the ESPGHAN guidelines removed classic symptoms, EMA-IgA positivity, and HLA DQ-2 or DQ-8 as crucial criteria for a diagnosis not based on biopsy[7]. However, EMA-IgA positivity is not discouraged[8,10]. The increasing confidence in diagnosing CD without biopsy in children has increased so rapidly that many recent studies consider tTGA > 10x as a new possible cut-off to further reduce the need for biopsies[52].

Recommendation		Changes over time
In children with classic symptoms, TGA-IgA titre > 10x, EMA-IgA positivity, and HLA DQ2/DQ8, the diagnosis can be achieved without a duodenal biopsy		Major changes over time
In children, classic symptoms, EMA-IgA positivity and HLA DQ2 /DQ-8 are not mandatory to diagnose CD if TGA-IgA titre is > 10x		Major changes over time
In adults, a diagnosis of CD without a positive biopsy is still discouraged		Exception Probable future changes over time 

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Figure 7 Recommendations about the possibility of a no-biopsy diagnosis. TGA: Anti-transglutaminase antibodies; IgA: Immunoglobulin A; EMA: Anti-endomysium antibodies; HLA: Human leukocytes antigen; CD: Celiac disease.

CD diagnosis without a positive duodenal biopsy has always been discouraged in adults[9-14]. This choice was not dictated by the reduced reliability of the serological tests in adults. In fact, large population studies concluded that tTG-IgA>10x could accurately predict villous atrophy[53]. Rather, other considerations currently prevent the extension of paediatric criteria into the adult population. First, CD at onset can be associated with complications. In the case of primary or secondary resistance, or slow response to the GFD, the absence of baseline histology may make the diagnosis of complications difficult[9]. Index histology may also predict the risk of future complications, such as lymphoma[54]. Moreover, endoscopy may help diagnose other treatable disorders associated with CD, such as eosinophilic esophagitis, autoimmune gastritis, and lymphocytic gastritis[9].

Both complicated CD and possible differential diagnoses of CD are virtually absent in children. However, they represent a serious concern in adults, thus justifying different diagnostic algorithms according to the age of presentation of the first symptoms.

Potential, silent and seronegative CD

Potential CD is characterised by a positive serology for CD in the absence of mucosal damage at biopsy[1]. As stated above, Marsh 1 Lesions (*i.e.*, an increased IELs count) are not suggestive of an active CD but may increase the risk of developing villous atrophy[41].

It is widely accepted that symptomatic potential CD may benefit from a GFD, and a direct challenge would be run[8-14]. In adult patients with both positive TGA-IgA and EMA-IgA CD is likely, and a GFD may be initiated irrespective of symptoms[9]. A serological response after a period of approximately 12 mo confirms the diagnosis of CD[9]. In EMA-IgA negativity, HLA-typing may exclude the diagnosis before embarking on follow-up[9]. If a follow-up is started, potential CD patients should be

retested after consuming a gluten-containing diet for 3-6 mo to confirm persistent seropositivity before referral for a new endoscopy (Figure 8)[9,10].

Silent CD is characterised by the presence of both positive serology and histology for CD in the absence of classical or non-classical symptoms[1]. It is widely recommended to start a GFD in patients with silent CD because it is considered an active form of the disease[8-14].

Seronegative CD is characterised by the presence of active enteropathy and negative serology for CD, with no other causes, and with clinical and histological responses to a GFD[1,37]. In these cases, other causes of enteropathy should be excluded before embarking on the direct challenge of a GFD[37,55]. HLA-typing can also rule out the diagnosis of CD in seronegative enteropathies[9,14,37]. Finally, the direct challenge of a GFD is advised only in patients with seronegative enteropathy, positive HLA typing with no other causes. A documented histological response after 1-3 years of GFD is needed to confirm the diagnosis[9,14,37]. No major changes occurred over time in the management of seronegative CD[9,14].

Refractory and complicated CD

CD can be complicated by a persistent active form of the disease, independent of gluten intake, known as refractory CD (RCD)[1]. Other rare complications of CD can be neoplastic. Primarily, enteropathy-associated T-cell lymphoma (EATL) is a rare T-cell lymphoma associated with untreated CD. EATL has an abysmal prognosis and can occur primarily at diagnosis or as an evolution of RCD type 2[56]. Duodenal adenocarcinoma is possible, albeit less frequent in the CD population[57].

Refractory CD (RCD) is characterised by the persistence or recurrence of symptoms and signs of malabsorption, with documented villous atrophy, despite a strict GFD for more than 12 mo and in the absence of other causes[9-14]. No major changes occurred in this definition over time (Figure 9).

RCD can be primary (refractory at the time of the first diagnosis), or secondary (occurring after a period of response to the GFD)[1]. The first step in evaluating suspected RCD is to re-evaluate the initial diagnosis of CD by reviewing biopsies and serology tests obtained at the time of diagnosis[58]. The most common cause of GFD failure is inadvertent gluten ingestion[59].Therefore, evaluation by an expert dietitian should always be included[9,10,13,14]. Other associated or concomitant pathological conditions should be excluded before RCD diagnosis. These include lactose and fructose intolerance, small intestinal bacterial overgrowth, microscopic colitis, pancreatic insufficiency, and inflammatory bowel diseases[59,60]. All guidelines recommend this strategy[9,10,13,14].

RCD is further classified into type 1 (RCD-1) and type 2 (RCD-2)[1]. T-cell flow cytometry is the most reliable method for classifying RCDs. Aberrant T cells lose the normal surface markers CD3 and CD8 with preserved expression of intracytoplasmic CD3. In RCD-1, the percentage of aberrant T cells is below 20%, whereas in RCD-2, they represent more than 20% of the total IELs[58]. RCD-2 can be considered a pre-lymphoma or low-grade lymphoma[54]. T-cell receptor (TCR) g chain clonality analysis lacks sensitivity and specificity, and is of limited value in separating RCD-1 from RCD-2[54]. TCR analysis has been formerly indicated as a criterion for differentiating RCD-1 from RCD-2[11,13,14]. The latest ESsCD guidelines exclude TCR analysis in the RCD classification[9].

RCD-1 has an extremely high 5-year survival rate (> 90%)[54,59,60]. In RCD-1, the first-line therapy should be 'open-capsule' budesonide (OCB), 3 mg, 3 times a day[61]. Budesonide (open capsule or not) has been progressively accepted as the first-line therapy for RCD-1[9,11,13,14]. In the ACG 2013 guidelines, systemic steroids are considered the first-line therapy for RCD-1[14]. Second-line treatment for RCD-1 includes immunosuppressive drugs such as steroids (prednisone 0.5-1 mg/kg/day) and azathioprine (2-2.5 mg/kg/day)[60]. Most guidelines agree with this strategy[9,11,12]. Systemic steroids can also be considered as first-line treatment while waiting for a specialist's advice[12]. Infliximab may be the preferred biological therapy for second-line treatment of RCD-1[62]. Evidence is still weak, and only one guideline includes infliximab as an RCD-1 treatment[9].Withdrawing of immunosuppressive therapy after 2-3 years of complete response may be considered[9,54].

RCD-2 is rarer than RCD-1, has a much higher mortality rate, and treatment is less well defined. Systemic steroids or open-capsule budesonide should be the first choice for milder presentations. In severe cases, cytoreductive therapies such as cladribine and fludarabine or autologous hematopoietic stem cell transplantation should be chosen[59,60]. Guidelines are mostly aligned with this strategy[9,13,14]. Some guidelines also report azathioprine, 6-mercaptopurine, methotrexate, cyclosporine, and anti-TNF antibodies as possible therapies, but the data are weaker[11,13,14]. Not every

Recommendation		Changes over time
Children and adults with symptomatic potential CD responding to GFD may be considered CD patients, despite the absence of villous atrophy		Minor changes over time. Extensions
Children and adults with silent CD are considered CD patients and must be treated		No major changes over time
In adults, a seronegative CD diagnosis can be achieved with a direct challenge of a GFD in patients with villous atrophy with no other causes, negative serology tests and positive HLA typing. A follow-up biopsy of confirmation is required		No major changes over time

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Figure 8 Recommendations about potential, silent, and seronegative celiac disease. GFD: Gluten-free diet; HLA: Human leukocytes antigen.

guideline has raised the topic of RCD-2 treatment[10-14].

Transformation to enteropathy-associated T-cell lymphoma (EATL) is likely in RCD-2[59]. VCE, positron-emission tomography (PET), and magnetic resonance (MR) enterography can be useful in cases of suspected progression to EATL to assess the extent of the disease[63]. All guidelines advise the use of these tools in RCD-2 staging [9-14]. Severe RCD-2 and EATL may require surgery, chemotherapy, or bone marrow transplantation[64]. The former therapeutic strategies are mostly based on case reports, and only one guideline extensively discusses them[9].

Follow-up

Since CD is the only autoimmune disease with a known environmental trigger (*i.e.*, gluten), a periodical assessment of compliance to a GFD is essential[65]. Poor GFD compliance is not infrequent, and mucosal damage can persist despite negative serology and the absence of symptoms[66]. Follow-up is also essential for evaluating possible complications[54]. Osteoporosis and metabolic complications of GFD should also be evaluated during follow-up[67-69]. Suggested follow-up schedules are based on the frequency of complications, risk of GFD non-compliance, and reported quality of life[70].

Therefore, there is universal agreement on the necessity of long-term monitoring of patients with CD to assess the compliance and responsiveness to the GFD and allow early detection of complicated CD (Figure 10)[8-14]. Follow-up evaluations should be scheduled every 3-6 mo during the first year and then every 1-2 years[9-14]. In children, follow-up should continue until they reach their final height[9-11,14], focusing on normal growth and development[9,10,14].

There is disagreement about who should oversee follow-up. While most guidelines show no preference between primary care physicians, specialists, or dietitians[9-11,13,14], the NICE 2015 guidelines suggest that dietitians with expertise in CD may be best suited to carry out an annual follow-up[12]. However, on a general principle, all guidelines agree that newly diagnosed patients should be referred to a dietitian[9-14]. Some guidelines suggest that nutritionist counselling should coincide with medical visits during follow-up[10,13]. The inclusion of a dietitian assessment at diagnosis and

Recommendation		Change over time
Slow-responder CD is defined as the persistence of symptoms, signs and laboratory abnormalities despite at least 6–12 months of GFD. This term replaces the former "non-responsive CD"		Major change over time
Refractory CD is defined as the persistence/recurrence of malabsorption, with documented villous atrophy, despite a strict GFD for > 12 mo and absence of other causes		No major changes over time
T-cell flow cytometry is the most reliable method for classification refractory CD		Major changes over time
TCR-gamma chain clonality analysis lacks sensitivity and specificity, and it is of limited value		Major changes over time
Budesonide is recommended as first-line therapy for refractory CD type 1		Major changes over time
Second-line treatments for refractory CD type 1 includes steroids, azathioprine and infliximab		Major changes over time
Therapy for refractory CD type 2 is not supported by strong clinical data		Major changes over time

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Figure 9 Recommendations about refractory and complicated celiac disease. GFD: Gluten-free diet; TCR: T-cell receptor.

during follow-up was supported by clinical data[71]. Indeed, nutritional counselling could also help manage metabolic alterations, which frequently appear during the first years of the GFD[67].

All guidelines also provide information about the essential information that should be collected during follow-up evaluations. These evaluations should include a dietary interview, serology (TTG-IgA if normal IgA), and laboratory tests[9-14]. Laboratory tests should evaluate the presence of micronutrients malabsorption, including complete blood count, iron status, folate, vitamin B12, calcium, phosphate, vitamin D, and should monitor associated autoimmune conditions (thyroid-stimulating hormone and serum glucose) and liver disorders (aspartate aminotransferase/alanine amino-transferase)[9-11,13,14]. Normalisation of tTG-IgA levels do not predict full recovery of villous atrophy. In contrast, persistently positive serology 12 mo after GFD initiation is a strong indicator of gluten ingestion[72]. All guidelines were aligned with the interpretation of tTG-IgA levels during follow-up[8-14].

The inability of serology alone to predict mucosal healing automatically leads to consider the opportunity of repeating duodenal biopsies after the start of the GFD. While the general agreement is that follow-up biopsies are not mandatory in

Recommendation		Changes over time
In adults, follow-up should be scheduled every 3-6 month during the first year and then every 1-2 yr		No major changes over time
A normal TGA level at the follow-up does not predict recovery of villous atrophy		No major changes over time
On the contrary, persistently positive serology 12 mo after starting a GFD strongly suggests gluten contamination		No major changes over time
The follow-up should include at least a dietary interview, serology, and laboratory tests evaluating absorption.		No major changes over time
Follow-up biopsy is not universally recommended but may be reasonable after 2 yr of GFD in high-risk patients		Minor changes over time
In children, follow-up should be scheduled every 3-6 mo during the first year and then every year until the end of development		No major changes over time
Newly diagnosed patients should be referred to a dietitian for management		Minor differences
Primary care physicians or dietitians with experience in dealing with CD may take responsibility for the follow-up		Some differences
Follow-up should also include periodical bone densitometry, vaccinations and psychological support		Some differences

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Figure 10 Recommendations about follow-up of celiac disease. TGA: Anti-transglutaminase antibodies; GFD: Gluten-free diet.

asymptomatic patients on a GFD and without an increased risk of complications[9-14], the guidelines diverge regarding other points. Many guidelines consider it reasonable to repeat biopsy after 2 years of GFD to assess mucosal healing[9,11,14]. Other guidelines suggest repeating biopsies only for persistent symptoms or serological abnormalities after 12 mo of GFD[10,12,13]. A growing body of literature suggests that the risk of a complicated CD is higher in patients >40 years of age at the time of diagnosis or those with a classical presentation[54]. Some guidelines agree that repeating biopsies should be of interest in these selected populations[13,14].

Some guidelines also provide suggestions for further examinations to be performed during follow-up. According to the ECD and Russian guidelines, bone densitometry should be offered to every patient at the time of diagnosis and should be repeated after 3 years if abnormal, or 5 years if normal[9,11]. Other guidelines suggest performing

bone densitometry only in patients with a high risk of osteoporosis or those older than 55 years[12,13].

While there is a general agreement in recommending a pneumococcal vaccine[8-10, 12], the WGO2017 guidelines also recommend vaccinations against *Haemophilus influenzae typeB*, and *Meningococcus*, while other guidelines state that these vaccines have a less clear indication to be given to every patient with CD[9,11-13].

Mood disorders are another common problem in patients with dietary restrictions. Anxiety, depression, and fatigue may be associated with CD before and after diagnosis and can affect the quality of life[73]. In this context, most guidelines agree on advising patients to join CD support groups and associations[9,10,12,13]. Some of them also suggest that psychological support provided by a specialist may be offered[12,13].

Gluten-free diet

Gluten is a protein with high proline and glutamine content, primarily found in wheat. Rye and barley belong to the same tribe as wheat and are known to contain gluten. In contrast, oats are derived from a different tribe and do not contain pure gluten[1].

Uncontaminated oats are safe for almost all patients with CD, but a small percentage of patients may be sensitive to some oat cultivars[74] and should be monitored[9,10,12-14]. Some guidelines advise the initiation of a Gluten-free diet (GFD), excluding oats, and recently introduced them[10,13,14]. The Russian guidelines (2016)are against oat consumption in patients with CD because of the high risk of contamination[11]. Even if not stated, oat consumption would be safe in many countries, though it may be discouraged in developing countries where contamination could be widespread (Figure 11).

WHO guidelines on 'Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten' state that foods labelled as 'gluten free' should contain ≤ 20 parts per million (ppm) of gluten[75].

Patients should be instructed to avoid contaminating their gluten-free food by using separate cooking utensils and cooking surfaces[9,10]. At present, shared items can be safely used if thoroughly cleaned with soap and water between use[9,76].

The duration of breastfeeding and the timing of gluten introduction to the infant seem to have no impact on the risk of developing CD, even in those at high risk[77]. Therefore, there are no strict indications for gluten introduction in infant diets[9]. Formerly, it was advised to avoid either early or late gluten introduction in children at risk of CD[13].

Dermatitis herpetiformis (DH) is a bullous cutaneous disease triggered by gluten consumption like CD[1]. DH and CD often coexist and share the same treatment, GFD [9,10,13,14]. Interestingly, the ESsCD guidelines suggest that psoriasis could also benefit from GFD in the case of documented CD serology, even in the absence of mucosal damage[9].

DISCUSSION

Our comparative analysis of the currently adopted CD guidelines underlined differences in diagnostic aspects and the management of the follow-up. These differences mirror some relevant clinical points in both developing and developed countries.

First, the differences in the diagnostic process of CD are important. The possibility of a no-biopsy diagnosis has relevant repercussions in developing countries. Most guidelines are still cautious in this regard, with the WGO2017 guidelines being the only ones contemplating this possibility in geographical areas with a paucity of resources. As correctly underlined by these guidelines, some absolute recommendations may not be valid for developing countries where the availability of serology or endoscopy may be lacking[10]. CD seems to have a non-negligible prevalence in Asia and sub-Saharan Africa[77,78]. Especially in Russia and Central Asia, the prevalence of CD is very likely to be underestimated due to poor disease awareness among physicians and/or patients, limited access to diagnostic resources, inappropriate use or interpretation of the serological tests, absence of standardised diagnostic and endoscopic protocols, and insufficient expertise in histopathological interpretation[3]. Specific guidelines are lacking in these geographical areas[79]. In addition, the incidence of undiagnosed CD in children can be extremely high[80]. Knowing the high mortality and disability related to untreated CD in childhood, it would be advisable to develop specific protocols for specific geographical areas.

Recommendation		Change over time
The mainstay for treatment of CD is a strict GFD. which usually resolves both classical and non-classical manifestations		No major changes over time
Uncontaminated oat is safe for almost every patient. A small percentage of patients may be sensitive to oats and should be monitored		Minor changes over time
GFD should be initiated also in psoriatic patients with positive CD serology		Major changes over time
The duration of breastfeeding and the timing of gluten introduction have no impact on the risk of developing CD		Major changes over time

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Figure 11 Recommendations about the gluten-free diet for celiac disease.

The no-biopsy approach has been discouraged for a long time, especially in adults [13,14]. In contrast, most recent guidelines have incorporated the ESPGHAN 2012 recommendations for a no-biopsy approach in children[9,10]. The possibility of an outright extension of these criteria into the adult population still meets key obstacles. However, in an era during which the COVID-19 pandemic has caused a staggering drop in new CD diagnoses even in industrialised countries[81], ESPGHAN released the advice to lower the TGA-IgA threshold for diagnosing CD without biopsy[52]. Moreover, retrospective data on a possible no-biopsy approach in adults are increasing [53]. Prospective data will probably lead to the integration of such an approach to future guidelines over the next decade.

Second, the differences in follow-up recommendations reflect a relatively low interest in this topic in the past. Arguably, the search for more reliable diagnostic tools was the right priority in an era characterised by a severe under-diagnosis of CD .Nowadays, significant diagnostic delays can still occur in a minority of Central European children[82], with socioeconomically deprived children being more likely to be underdiagnosed despite improved and easily available serological testing[4].

Nonetheless, the current physicians' awareness of CD has reached fairly high levels, and the case-detection strategy has significantly contributed to the increased number of diagnoses. Consequently, the correct management of follow-up is crucial. This topic is of special interest in developed countries, in which metabolic problems possibly caused by an unbalanced GFD are particularly prevalent. Uncontrolled weight gain, metabolic syndrome, and non-alcoholic fatty liver disease are epidemic in these countries and can also be facilitated by the GFD[67,69,83-85]. In addition, quick detection of associated autoimmune conditions can prove highly beneficial, especially in autoimmune liver diseases[86]. Finally, early detection of complicated CD requires particular attention, as both neoplastic and non-neoplastic complications may arise years after the diagnosis[6].

CONCLUSION

We found a relatively high concordance between CD guidelines. Important modifications have occurred in recent years, especially regarding the possibility of a no-biopsy diagnosis in children. Other modifications are expected in the future and will probably involve the extension of the non-invasive diagnosis to the adult population and the follow-up modalities.

ARTICLE HIGHLIGHTS

Research background

Celiac disease (CD) has risen from obscurity to global prominence in a few decades. These modifications have prompted experts from all over the world to identify effective strategies for the diagnosis and follow-up of CD. Different scientific societies, mainly from Europe and America regions, have proposed different guidelines.

Research motivation

CD guidelines are consistent when they deal key points in the diagnosis and follow-up of this condition. However, they differ in a number of other points.

Research objectives

To identify all of the existing guidelines across the globe and perform a comparative analysis to verify similarities and differences and, thus, discuss the most debated topics and the possible innovations in the next future.

Research methods

We searched PubMed for a complex string containing the terms "celiac disease", "management", and "guidelines". The results were subsequently explored to identify the most recent versions of existing guidelines of governmental agencies and scientific societies. The recommendations provided by each selected guideline were systematically explored and classified under five categories: Patients to be tested for CD, diagnostic tests (serology, duodenal biopsy, genetic test, no-biopsy diagnosis), potential/silent/seronegative CD, refractory/complicated CD, follow-up.

Research results

We identified 7 different guidelines [European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020; European Society for the Study of Coeliac Disease (ECD) 2019; World Gastroenterology Organization (WGO) 2017; Central Research Institute of Gastroenterology, Russia, 2016; National Institute for Health and Care Excellence (NICE), 2015; British Society of Gastroenterology (BSG), 2014; and America College of Gastroenterology (ACG), 2013]. These guidelines were mostly concordant but differed under certain recommendation for no-biopsy diagnosis, refractory CD, and follow-up.

Research conclusions

We found a relatively high concordance between the guidelines for CD. Important modifications have occurred in the last years, especially about the possibility of a no-biopsy diagnosis in children.

Research perspectives

Modifications of the current guidelines are expected in the near future. These modification will probably regard the possibility of a no-biopsy diagnosis (especially in developing countries) and the modalities of follow-up.

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Contents

Weekly Volume 28 Number 2 January 14, 2022

REVIEW

176 Nanotheranostics: A powerful next-generation solution to tackle hepatocellular carcinoma

Ladju RB, Ulhaq ZS, Soraya GV

MINIREVIEWS

188 Multiple subcellular localizations and functions of protein kinase Cδ in liver cancer

Yamada K, Yoshida K

199 Therapeutic endoscopy for the treatment of post-bariatric surgery complications

Larsen M, Kozarek R

216 Update on the applications and limitations of alpha-fetoprotein for hepatocellular carcinoma

Hanif H, Ali MJ, Susheela AT, Khan IW, Luna-Cuadros MA, Khan MM, Lau DTY

ORIGINAL ARTICLE

Case Control Study

230 Obesity is associated with decreased risk of microscopic colitis in women

Sandler RS, Keku TO, Woosley JT, Sandler DP, Galanko JA, Peery AF

Observational Study

242 Identification of functional tumor necrosis factor-alpha promoter variants associated with *Helicobacter pylori* infection in the Sudanese population: Computational approach

Idris AB, Idris AB, Gumaa MA, Idris MB, Elgoraish A, Mansour M, Allam D, Arbab BM, Beirag N, Ibrahim EAM, Hassan MA

Prospective Study

263 Outreach onsite treatment with a simplified pangenotypic direct-acting anti-viral regimen for hepatitis C virus micro-elimination in a prison

Chen CT, Lu MY, Hsieh MH, Tsai PC, Hsieh TY, Yeh ML, Huang CI, Tsai YS, Ko YM, Lin CC, Chen KY, Wei YJ, Hsu PY, Hsu CT, Jang TY, Liu TW, Liang PC, Hsieh MY, Lin ZY, Huang CF, Huang JF, Dai CY, Chuang WL, Shih YL, Yu ML

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Contents

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REVIEW

Nanotheranostics: A powerful next-generation solution to tackle hepatocellular carcinoma

Rusdina Bte Ladju, Zulvikar Syambani Ulhaq, Gita Vita Soraya

ORCID number: Rusdina Bte Ladju 0000-0001-5393-0429; Zulvikar Syambani Ulhaq 0000-0002-2659-1940; Gita Vita Soraya 0000-0002-2656-4176.

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Rusdina Bte Ladju, Department of Anatomic Pathology, Faculty of Medicine, Hasanuddin University, Makassar 90245, Indonesia

Zulvikar Syambani Ulhaq, Department of Biochemistry, Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim Islamic State University, Malang 65151, Indonesia

Zulvikar Syambani Ulhaq, National Research and Innovation Agency, Central Jakarta 10340, Indonesia

Gita Vita Soraya, Department of Biochemistry, Faculty of Medicine, Hasanuddin University, Makassar 90245, Indonesia

Gita Vita Soraya, Department of Neurology, Faculty of Medicine, Hasanuddin University, Makassar 90245, Indonesia

Corresponding author: Gita Vita Soraya, MD, PhD, Assistant Professor, Department of Biochemistry, Faculty of Medicine, Hasanuddin University, Jl Perintis Kemerdekaan Km 10, Makassar 90245, Indonesia. gitavitasoraya@unhas.ac.id

Abstract

Hepatocellular carcinoma (HCC) is an epidemic burden and remains highly prevalent worldwide. The significant mortality rates of HCC are largely due to the tendency of late diagnosis and the multifaceted, complex nature of treatment. Meanwhile, current therapeutic modalities such as liver resection and transplantation are only effective for resolving early-stage HCC. Hence, alt-ernative approaches are required to improve detection and enhance the efficacy of current treatment options. Nanotheranostic platforms, which utilize biocompatible nanoparticles to perform both diagnostics and targeted delivery, has been considered a potential approach for cancer management in the past few decades. Advancement of nanomaterials and biomedical engineering techniques has led to rapid expansion of the nanotheranostics field, allowing for more sensitive and specific diagnosis, real-time monitoring of drug delivery, and enhanced treatment efficacies across various malignancies. The focus of this review is on the applications of nanotheranostics for HCC. The review first explores the current epidemiology and the commonly encountered obstacles in HCC diagnosis and treatment. It then presents the current technological and functional advancements in nanotheranostic technology for cancer in general, and then specifically explores the use of nanotheranostic modalities as a promising option to address the key challenges present in HCC management.

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Core Tip: Hepatocellular carcinoma (HCC) is a global epidemic burden. The high mortality rate is mostly due to late diagnosis and complexity of treatment. Nanotheranostics is a potential approach for HCC management. We herein discuss the challenges of HCC management, the advancement of nanotheranostics in cancer, and the potential role of nanotheranostics to address the current challenges in HCC management.

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INTRODUCTION

Epidemic burden and risk factors of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and remains the second leading cause of cancer-related deaths worldwide[1,2]. It accounts for around 90% of all primary liver cancer cases worldwide[3], and is an epidemic burden in both developing and developed countries[4]. Whilst certain endemic areas such as East Asia has shown a decreasing trend, regions such as Europe, Africa, and the United states display increasing trends in HCC incidence rate with substantial morbidity and mortality[5,6]. Concerningly, cases have doubled in Europe and America as a result of lifestyle factors such as alcohol abuse, smoking, obesity, and metabolic diseases[7-10]. Variations among age and gender are also interesting epidemiologic features of HCC. Men have a higher prevalence of HCC than women with a ratio of 462.4:185.8 new cases per year in developing countries[11]. Respectively, the risk of HCC significantly increases among those who are older than 40 years of age[12].

The risk factors and etiologies of HCC vary depending on geographic region and lifestyle. Hepatitis B and C infections are major etiological factors that significantly contribute to HCC globally[13-15], accounting for 44% and 21% of HCC cases respectively[16], with the highest number of hepatitis B cases occurring in Asia. Other possible risk factors include the increasing number of nonalcoholic fatty liver disease (NAFLD)[17], alcohol addiction[18,19], and aflatoxin consumption[20]. In Western, Central, and Eastern Europe and North America, a majority of HCC cases were attributed to NAFLD/non-alcoholic steatohepatitis, obesity, and excessive alcohol consumption. In contrast, most HCC cases in Asia and Africa were attributed to hepatitis B virus infection[21,22]. The viral and metabolic etiologies described above not only contributes to HCC occurrence, but also implicates a high risk of de novo recurrence, leading to the development of incurable and advanced stage disease that is resistant towards therapeutic efforts such as complete tumor resection or ablation[6].

Pathological complexity of HCC

Due to the multifactorial nature of HCC, several cellular phenomena can be observed, including hypoxia, inflammation, oxidative stress, and tumor microenvironment. Indeed, the molecular mechanism of liver carcinogenesis involves multiple endogenous and exogenous genetic alterations[23]. Hepatocarcinogenesis is a deliberate and complex multistep process associated with somatic genomic alterations, leading to the production of cellular intermediates that progress into hepatocellular carcinoma [24]. The development of HCC involves a combination of continuous inflammatory damage, necrosis, and fibrotic deposition. The pre-neoplastic stage is a long process that typically requires 10 to 30 years of time. During this stage, phenotypically altered hepatocytes are formed as a result of either DNA methylation alterations, pathogenic agent's reaction, and point mutation or loss of heterozygosity, which occur in part

through epigenetic mechanisms that lead to the development of dysplastic hepatocytes in foci and nodules. Aberrant and dysplastic hepatocytes are related to the accumulation of permanent structural alteration and changes in genes and chromosomes[25]. Alterations in the malignant phenotype are often distinct, suggesting heterogeneity at the genomic level[25,26].

Challenges in HCC diagnosis

Early diagnosis is a major challenge in HCC management, and in most cases, the lack of early diagnostic modalities lead to less than optimal treatment outcomes. In developed countries, 30%-60% of HCC cases can be diagnosed early, enabling higher success rates of curative treatment. Contrastingly, HCC cases in developing countries are mostly diagnosed in late stages, leading to substantially lower likelihood of curative treatment[27]. Diagnosis of HCC is in fact, an important and critical phase that relates directly to the survival and prognosis of the patients.

Complexity of HCC treatment

Treatment of HCC itself is also complex and multifaceted, and outcome depends on the time of diagnosis and the presence of additional comorbidities. Prompt diagnosis of HCC is correlated with better outcomes of curative therapies. This is demonstrated by studies that show higher efficacy of local radiofrequency ablation and surgical intervention (liver transplantation and liver resection) in the very early and early-stage HCC as compared to later stages[8,28]. However, most HCC patients are excluded from definitive surgical resection due to late diagnosis. In such cases, liver transplantation can be the best treatment for HCC with a low risk of recurrence, though it is suggested as a second line treatment due to the disparity between limited liver donor resources and the increasing number of patients[29,30]. Based on international guidelines, late stage HCC patients (intermediate and advanced) may receive palliative treatment such as chemoembolization and systemic therapy, while terminal patients can only receive supportive care[27,28,31].

Issues such as tumor recurrence and drug resistance are also major obstacles that frequently complicate HCC management[32,33]. The 5-year recurrence probability of HCC is around 62% after liver ablation[34] and 80% after liver resection[35]. Palliative treatment often have unexpected and poor outcomes related with high refractoriness to systemic therapy that lead to development of multidrug resistance[36-38].

Need for a novel approach in HCC management

The challenges associated with both diagnosis and treatment of HCC has resulted in the high mortality rates across the globe, and calls upon innovative approaches that can improve the prognosis of HCC patients. In the following sections, we describe the rapid advances and implementation of theranostic-based nanomedicine and nanoparticles (nanotheranostic) as a promising option (Figure 1) for the improvement of HCC patient outcomes and quality of life.

POTENTIAL OF NANOTHERANOSTICS FOR PRECISION CANCER MEDICINE

Nanotheranostic modalities present a promising solution to the diagnostic and therapeutic challenges encountered in HCC management, through the use of biocompatible nanoparticles that simultaneously performs both diagnostic and therapeutic functions. This approach potentially provides a more personalized and targeted approach to cancer therapy, wherein the nanoparticles can be designed to detect specific biomarkers of the target malignant region, allow real-time monitoring or visualisation of the target, and finally deliver therapeutic modalities in a more precise manner. In recent years, the nanosensor and nanomedicine technologies have experienced major development, and have paved the way for promising means of nanotheranostics implementation in cancer management.

Nanotheranostic is a real-time combination of novel therapeutic and modern diagnostic tool or imaging into a single agent linked and integrated by nanoparticles [39,40]. Nanoparticles are the key components of the nanotheranostic agent[41] which include aptamer[42], DNA nanostructure[43], lactosome-based nanoparticles[44], metallic nanoparticles[45], gold nanoparticle[46], silver nanoparticle[47], dendrimer and copolymer-based nanoparticles[48], lipid-based nanomaterials[49], magnetic nanoparticles[50] iron oxide nanoparticle[51], mesoporous silica nanoparticle[52] and

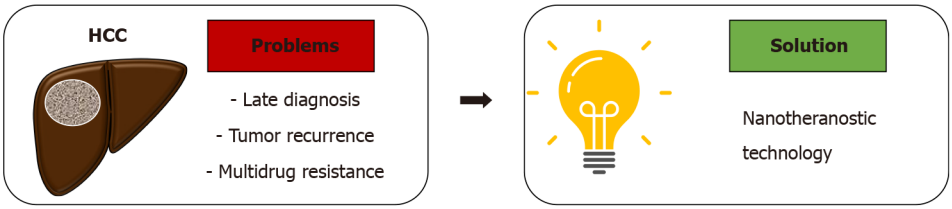


Figure 1 Hepatocellular carcinoma current problems and solution. HCC: Hepatocellular carcinoma.

quantum dots nanoparticle[53].

Nanotheranostic is an ideal choice for cancer treatment in the era of personalized medicine due to its potential to overcome the diagnostic and therapeutic challenges described prior[54]. Nanotheranostic not only provides the means for early diagnostic tools[55], nanoimaging-therapeutic integrated medicine[56], targeted-therapy[57] and tumor-specific nano-delivery agent[58], it also holds potential for real-time monitoring of drug response, and reduce side effects and drug toxicity in patients[59,60] as shown in Figure 2.

Successful demonstration of nanotheranostics for diagnostics and targeted therapy has been shown by Roy *et al*[61] in which highly sensitive, polymer-modified gadolinium-doped iron oxide-based T_1 contrast agents were used for successful methotrexate drug delivery. In the second application, nanotheranostics have been utilized for simultaneous imaging and cancer monitoring[62]. An auto-fluorescent platform, constructed from a positively charged amphiphilic polymer polyethyleneimine-polylactide, was utilized to simultaneously load the antiangiogenesis agent cobretrastatin together with near-infrared (NIR) dye IR825 and heat-shock protein inhibitors. Altogether, the mechanism represents self-monitoring nanotheranostics, which in a mouse model demonstrated inhibitive properties in the tumour site through anti-angiogenesis and gene silencing enhanced photothermal therapy, while allowing real-time fluorescence monitoring.

The final and most widely developed application of nanotheranostics is for simultaneous imaging and targeted therapy, which has been shown to substantially increase the overall efficacy of therapies[63-65]. Theranostic platform choice has expanded rapidly in the past decade, and typically combines imaging modalities such as magnetic resonance imaging (MRI), NIR fluorescence, photoacoustic (PA) or ultrasound imaging, with therapeutic modalities such as chemotherapeutic agents, x-rays, hyperthermia, or free radicals.

Depending on the desired diagnostic and therapeutic modality, the nanoparticle of choice may be composed of metals, polymers, carbons and lipids. Each choice provides its own unique characteristics and physicochemical interactions, and also require different fabrication and functionalization procedures. As an example, successful magnetic-based imaging in a nanotheranostic platform is achievable using iron oxide, which is also desired due to its low toxicity and chemical stability. But many platforms prefer the use of multi-functional semiconducting polymers with hydrophobic properties, which simultaneously allow imaging through easy interactions with aromatic chemotherapeutic agents[66]. Nanoparticles can also be engineered to provide multimodal imaging[67] which utilizes modified ultrasmall Ag_2Se nanodots to allow upconversion luminescence, downshifting luminescence, computed tomography and PA imaging techniques.

An increasingly common approach in cancer theranostics is the use of multimodal therapy. To illustrate, the study by Zhang *et al*[68] utilized Janus-type γ-Fe_2O_3/SiO_2 nanoparticles to combine the glucose oxidase-mediated cancer starvation strategy with hydroxyl radicals as chemodynamic therapy. Interestingly, nanoparticles can also be designed to become responsive towards environmental stimuli in drug-resistant tumours, meaning that it can be developed specifically towards the pathological profile of the tumor microenvironment as well as the organ-specific tissues and compartments, which contribute to the overall specificity of the drug delivery. For highly complex pathologies such as HCC with drug resistance, this provides a myriad of options for exploration, and becomes an interesting approach for future implementation of HCC-specific nanotheranostics.

Finally, it is also worth noting that metastasis remains a major issue in cancers such as HCC where diagnosis tends to be late. An interesting strategy[69] showed the use of immunotherapy-based theranostics to specifically target metastatic tissue. In said study, magnetic-responsive immunostimulatory nanoagents were added with

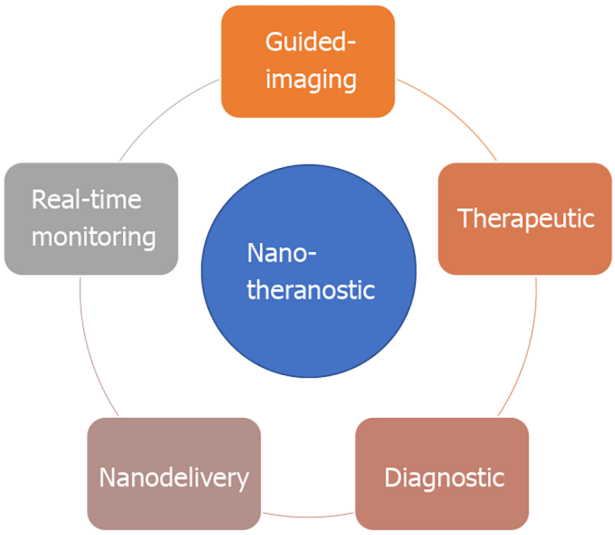


Figure 2 Applications of cancer nanotheranostic.

superparamagnetic iron oxide nanoparticles and cytosine-phosphate-guanine oligodeoxynucleotides. These engineered components allow for PA and MRI in addition to acting as a therapeutic agent for photothermally triggered immunotherapy.

To illustrate these advancements, we present the current modalities of cancer nanotheranostics in Table 1. In general, cancer nanotheranostics has been used for simultaneous diagnostics and therapeutic[70], real-time monitoring of malignancies [71], guided-imaging[72,73], drug-delivery[74] and multimodal-targeted therapy[75-79].

TACKLING HCC WITH NANOTHERANOSTICS

The nanotheranostic platform is a promising approach that is urgently needed to overcome the limitations of conventional therapy and diagnosis for more efficient HCC management. Figure 3 illustrates the multimodality of the nanotheranostic platform, which utilizes multipurpose nanoparticles for targeted nano-delivery, continuously controlled release of anticancer agents, guided imaging and early detection, for superior effectivity of transport[80]. Management of HCC requires powerful theranostic-based nanoparticles for early diagnostics and therapeutics with higher sensitivity and specificity, and to surpass the limitation of tissue penetration [81]. Previous studies have demonstrated the promising potential of silica-based nanomaterial as a potent nanotheranostic platform of HCC targeted-therapy nano-delivery[82-86]. In addition, many advances in HCC-specific nanotheranostics platforms are illustrated in Table 2, which demonstrates the multifunctional role of nanotheranostics as a detector to identify the HCC cell and tumor inhibitor by suppressing proliferation, migration and invasion of HCC[87,88].

One of the most remarkable advancements of the nanotheranostic platform is imaging and nano-delivery integration as an innovative resolution for early HCC diagnosis and *in situ* drug release. *In vivo* and *ex vivo* investigations have observed specific nanoplatform activation by the tumor, with minimized toxicity towards non-target cells[89]. Integration of multifunctional nanoparticles with MRI may provide novel perspectives in tumor imaging technology to enhance HCC management and treatment strategy. High precision quantification and sensitivity of nanoimaging technology is needed for tissue penetration issue in early diagnosis of HCC[90].

Aptamer-based nanotheranostic is also a potential tool for HCC management due to its unique characteristics. This oligonucleotide nanomedicine has high specificity and affinity towards various types of target molecules[91]. In HCC clinical application, aptamer-based nanotheranostic development targeting the epithelial cell adhesion molecule demonstrated an improvement of MRI application and drug-delivery with high efficiency of doxorubicin released specifically towards cancer cells[92].

Improvement of therapeutic success is urgently needed for patients with unresectable and advanced HCC. The combination between nanomedicine as a nano-

Table 1 Cancer nanotheranostics

Applications	Principle	Ref.
Diagnostic and therapeutic	Stimuli responsive nanoparticle and targeted drug delivery	[61]
	Activatable nanotheranostic systems diagnosis and therapy of peritoneal metastasis	[70]
Real-time monitoring and therapeutic	Self-monitoring and triple-collaborative therapy *via* auto-fluorescence nanoparticles	[62]
	Real-time monitoring and tumor targeting *via* dual-fluorescent hydroxyapatite–doxorubicin	[71]
Guided-imaging and nanodelivery	Nanoparticle conjugated with antibody for tumor targeting and guided drug delivery	[63]
	A protein-stabilized multifunctional nanoplatform for multimodal imaging and drug-delivery	[64]
Guided-imaging and therapeutic	Dual-targeting nanotheranostic with chemosensitizing agent for MDR chemotherapy	[65]
	Multifunctional nanocarrier for fluorescence imaging guided chemo-photothermal	[66]
	Dual-modal imaging and synergistic cancer starvation/chemodynamic therapy	[68]
	Tetra-modal imaging guided photothermal therapy	[67]
	Bimodal imaging guided photothermal-triggered immunotherapy	[69]
	Hierarchical tumor acidity-responsive magnetic nanobomb photodynamic therapy	[73]
	Lipid based nanoparticles nanodelivery-anticancer drug and nanoimaging	[74]
	The self-assembly nanoparticles with guided imaging and chemotherapeutic drugs	[76]
	Biocompatible nanoparticles as targeted-nanodelivery of chemotherapeutic agent	[77]
	Dual-modality mapping guided photothermal ablation for metastatic cancer	[78]
	Magnetic nanoparticle-doxorubicin for enhancing nanoimaging and targeted therapy	[79]

MDR: Multidrug resistance.

Table 2 Nanotheranostic development againts hepatocellular carcinoma

Applications	Principle	Ref.
Diagnostic and therapeutic	Conventional SELEX	[87]
	CE-SELEX	[88]
	Magnetic nanoparticle-aptamer	[92]
Enhancing therapeutic	Inducing tumor regression using siRNA-nanoparticle construction	[100]
	Enhancing the anticancer efficacy using siRNA-nanoparticle construction	[101]
	Enhancing chemotherapy using microRNA 375-nanoparticle construction	[102]
	Synergistic antitumor effect of microRNA 375-nanoparticle construction	[103]
Diagnostic and guided-imaging	'Activatable' aptamer-based fluorescence probe	[104]
	Streptavidin-fluorescent silica nanoparticles combination	[105]
	Aptamer- based electrochemical biosensors	[106]
Gene editing	Next-generation CRISPR/Cas technology	[107]

HCC: Hepatocellular carcinoma; SELEX: Systematic Evolution of Ligands by Exponential Enrichment.

delivery system with cancer immunotherapy holds great potential for enhancing the nanotherapeutic outcome for this population. A promising targeted-nano-delivery immunotherapy for advanced HCC that is currently undergoing clinical trial is the 4th generation chimeric antigen receptor (CAR) T cells targeting glypican-3 (GPC3) (ClinicalTrials.gov Identifier: NCT03884751)[93]. This study showed promising phase I results in regard to antitumor activity and safety profile of CAR-GPC3 T-cell immunotherapy. The antitumor activity is positively associated with tumor response with no grade 3/4 neurotoxicity effect in any patients[94]. Several studies have also been done

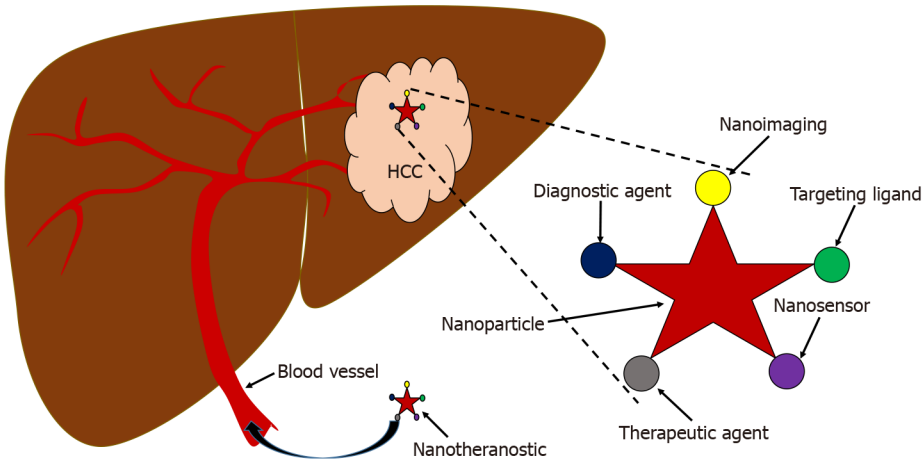


Figure 3 Multimodality of the nanotheranostic platform. HCC: Hepatocellular carcinoma.

to achieve said goal by conjugating anticancer drugs with nanoparticles, rendering the treatment safer with more effective systemic administration due to the platform's capability of controlling and postponing drug release. In the *in vivo* mouse model, tumor specific uptake of the controlled drug release for several weeks was observed, with minimal toxicity[95].

Molecular-targeted nano-therapies have also been constructed for nano-delivery using a modular design of polymeric nanoparticles for selective accumulation of drug pay load within tumor lesions. In *in vivo* mouse models, the intravenous drug injection was more effective for tumor inhibition than oral administration. This has revolutionized anticancer therapy by enhancing the efficacy and potency of therapeutics through inhibition of the angiogenesis pathway, tumor growth, tube formation and metastasis[96]. Targeted drug delivery using mesoporous silica nanoparticle is also promising. Nanoconstruction of silica nanorattle encapsulated docetaxel exhibited low toxicity with high antitumor activity, making it a prospective candidate for nano-delivery system[97]. Moreover, modified silica nanoparticles targeting low density lipoprotein and loaded with two anticancer drugs for liver cancer chemotherapy showed increased delivery efficiency based on *in vitro* and *in vivo* analysis[98].

In addition to anticancer drug nano-delivery for HCC treatment, the nanotheranostic platform is also suitable for targeted nano-delivery of small interfering RNA based therapeutics. This can be used as gene therapy to knock down a specific gene [99-101], and micro RNA for enhancing chemotherapy efficiency[102] to overcome multi-drug resistance in HCC[103].

FUTURE PERSPECTIVES

HCC is an extremely complex and heterogeneous disease with diverse molecular profiles, aetiology and subtypes. Since conventional approaches still fail to overcome limitations in HCC management, nanotheranostic is a promising alternative to overcome the problems. Rapid development in nanotechnology has added a tremendous value on cancer therapy. The future of cancer nanomedicine lies on multimodal nanoplatforms that combine targeting ligands, imaging agents, diagnostic agents and therapeutic components into one unit of functionalized nanoparticles. Thus, multifunctionality is a powerful and unique advantage of nanotheranostic over traditional methods, and evidence has shown its capacity to work efficiently and noninvasively *in vivo* without systemic toxicity. Development of nanotheranostic in the right direction requires improvement of platforms so that it can be optimized simultaneously for proficient performance as the best clinical outcome in HCC.

CONCLUSION

In summary, nanotheranostic is an emerging and promising approach for HCC diagnosis/imaging and therapy in the future. Nanotheranostic is a powerful, unique, and multifunctional tool that yields positive impact both in the basic research and

clinical application of HCC. We predict that in a near future nanotheranostic platform will continue to exponentially grow and progressively implemented in the development of novel and efficacious diagnostic and therapeutic agents towards cancers, including HCC. Further expansion would be needed to assist clinical translation of the promising preclinical studies in HCC.

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MINIREVIEWS

Multiple subcellular localizations and functions of protein kinase Cδ in liver cancer

Kohji Yamada, Kiyotsugu Yoshida

ORCID number: Kohji Yamada 0000-0001-9493-5269; Kiyotsugu Yoshida 0000-0003-3108-7383.

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Kohji Yamada, Kiyotsugu Yoshida, Department of Biochemistry, The Jikei University School of Medicine, Tokyo 105-8461, Japan

Corresponding author: Kiyotsugu Yoshida, MD, PhD, Professor, Department of Biochemistry, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. kyoshida@jikei.ac.jp

Abstract

Protein kinase Cδ (PKCδ) is a member of the PKC family, and its implications have been reported in various biological and cancerous processes, including cell proliferation, cell death, tumor suppression, and tumor progression. In liver cancer cells, accumulating reports show the bi-functional regulation of PKCδ in cell death and survival. PKCδ function is defined by various factors, such as phosphorylation, catalytic domain cleavage, and subcellular localization. PKCδ has multiple intracellular distribution patterns, ranging from the cytosol to the nucleus. We recently found a unique extracellular localization of PKCδ in liver cancer and its growth factor-like function in liver cancer cells. In this review, we first discuss the structural features of PKCδ and then focus on the functional diversity of PKCδ based on its subcellular localization, such as the nucleus, cell surface, and extracellular space. These findings improve our knowledge of PKCδ involvement in the progression of liver cancer.

Key Words: Protein kinase Cδ; Liver cancer; Subcellular localization; Tumor suppression; Tumor progression

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Core Tip: Protein kinase Cδ (PKCδ) plays multifunctional roles in various cancers, including liver cancer. PKCδ has been shown to exert pleiotropic functions through various stimuli responsiveness, post-translational modifications, and subcellular localization. Recently, we found that PKCδ is secreted extracellularly and resides on the cell surface of liver cancer cells, which contributes to tumorigenesis. In this review, we focus on the localization of PKCδ to discuss its characteristic localization patterns and functions in liver cancer, and outline the involvement of PKCδ localized intra- and extracellularly with distinct functions in the progression of liver cancer.

quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

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INTRODUCTION

The protein kinase C (PKC) family of serine/threonine kinase proteins in mammals, comprising the classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) subfamilies, is one of the defining families of AGC kinases[1,2]. To date, 10 isoforms of PKC have been identified in humans, including four cPKCs (PKCα, -βI, -βII, and -γ), four nPKCs (PKCδ, -ε, -η, and -θ), and two aPKCs (PKCζ and -λ/ι)[2,3]. PKC activation depends on the conformational activation of certain intracellular factors. Notably, PCK activation is regulated not only by binding to lipid factors, such as diacylglycerol (DAG) and phorbol esters, but also by protein phosphorylation[4]. PKCδ is often phosphorylated at several Tyr residues by various types of stimulations, including DNA-damaging reagents and oxidative stress[5,6]. Kinases that phosphorylate PKCδ at Tyr include the Src family of tyrosine kinases (*e.g.*, Src, Fyn, Lyn, and Lck) and c-Abl [6].

Among PKC families, PKCδ is a unique non-signal peptide-containing intracellular protein that has been reported to translocate to a diverse range of distributions, including the cytosol, nucleus, endoplasmic reticulum, Golgi, mitochondria, and plasma membrane, in response to different stimuli and cell types[8]. For example, a nuclear localization signal (NLS) was identified in the catalytic domain of PKCδ, which is necessary for the transport of PKCδ across the nuclear pore. Nuclear localization of PKCδ is associated with pro-apoptotic functions. Phosphorylation also affects the subcellular localization of PKCδ and its activation. Our recent study revealed that cytosolic PKCδ translocates to the extracellular space and acts as a growth factor for liver cancer cells or tumors[9]. In this review, we summarize studies reported to date regarding the intracellular function of PKCδ in cancerous phenotypes of liver cancer. We then focus on and discuss the relationship with subcellular localizations, which exist in extracellular and intracellular locations, and the functions of PKCδ. Increased knowledge on where PKCδ protein is localized and how it functions in living cells allows a more profound understanding of the functional diversity of PKCδ.

STRUCTURAL FEATURES OF PKCδ

PKCδ comprises an N-terminal regulatory domain and a C-terminal kinase core domain[5]. The C-terminal catalytic domain of PKC is conserved between isoforms and includes ATP- and substrate-binding sites and a kinase core[10,11]. The N-terminal regulatory domain is much less conserved and contains specific motifs for each isoform that are activated in response to unique signals. The regulatory modules in this N-terminal domain include the pseudosubstrate motif and C1 and C2 domains, which bind to Ca^{2+} and DAG. The affinity of the C1 and C2 domains for Ca^{2+} and DAG determines the cofactor requirements for the activation of specific PKC isoforms. cPKCs have functional C1 and C2 domains that bind to both Ca^{2+} and DAG[12-14], whereas nPKCs have a functional C1 domain that binds to DAG alone and a non-functional C2 domain, rendering these kinases independent of Ca^{2+} for activation[15].

Generally, upon PKC activation, growth factors and G protein-coupled receptors trigger the hydrolysis of membrane lipids by recruiting phospholipase C[16,17]. Phospholipase C generates DAG and inositol-1,4,5-triphosphate through the hydrolysis of membrane phosphoinositol. In response to DAG, PKC is translocated to the lipid membrane *via* the C1 domain, enabling interaction with its substrates and the phosphorylation response[18].

PKCδ confers distinct allosteric regulation *via* protein binding to the C1, C2, and V5 domains, tyrosine phosphorylation, and the removal of the regulatory domain by caspase cleavage (Figure 1). In particular, various reports have identified that a variety of tyrosine phosphorylation sites affect cellular functions. For example, many studies have demonstrated that tyrosine phosphorylation of PKCδ plays a critical role in cell death in response to apoptotic stimuli. Tyrosine residues important in the context of

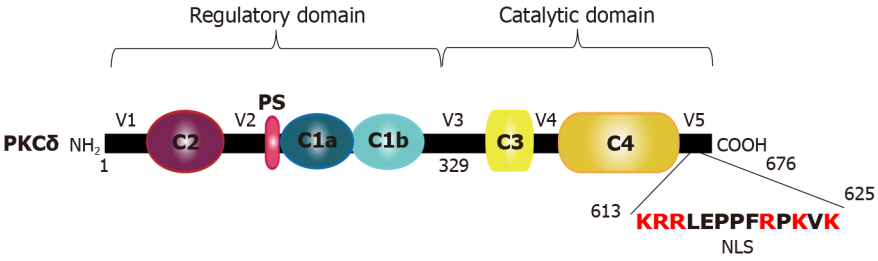


Figure 1 Schematic representation of protein kinase Cδ domains. The N-terminal regulatory domain is composed of 1 to 329 amino acids, non-functional C2, pseudosubstrate, and lipid binding C1 (a and b) domains. The C-terminal catalytic domain is composed of 330 to 676 amino acids, ATP-binding C3, and kinase C4 domains. The 329 amino acids at the V3 region allow the cleavage site by caspase-3 to be constitutively active. The V5 region includes nuclear localization signal necessary for the nuclear transport of protein kinase Cδ. NLS: Nuclear localization signal.

apoptosis include Tyr64, Tyr155, Tyr187, Tyr311, Tyr332, and Tyr512[18-20]. Furthermore, tyrosine phosphorylation of Tyr64 (C2 domain) and Tyr155 (C1a domain) is crucial for the exposure of PKCδ to NLS by disrupting the association between C2 and catalytic domains to enable nuclear transport.

FUNCTIONAL FEATURES OF INTRACELLULAR PKCδ IN LIVER CANCER

Tumor suppressive function

Studies on PKCδ$^{-/-}$ mice have confirmed the pro-apoptotic role of this molecule in response to several stimuli, such as DNA damage. Although these mice developed normally and were fertile, increased B cell proliferation was observed[21]. Smooth muscle cells derived from PKCδ$^{-/-}$ mouse aortas were also shown to be resistant to cell death in response to several stimuli. Hence, these studies with PKCδ$^{-/-}$ mice demonstrated that PKCδ is not required for cell proliferation during development.

PKC also binds to and is activated by tumor-promoting phorbol esters[4]. Therefore, PKC is considered a tumor-promoting protein. However, it has been reported that persistent treatment with phorbol esters causes degradation or downregulation of PKC [3,22-24]. In particular, PKCδ has been reported to enhance ubiquitin proteasomal degradation upon activation of PKCδ by lipids. Furthermore, accumulating evidence on PKCδ in cancer has shown that downregulation, rather than activation, of PKCδ is associated with tumor progression. Therefore, PKCδ is believed to act as a tumor suppressor because its downregulation facilitates tumor promotion and causes cell cycle arrest or induces apoptosis in response to various stimuli, such as H_2O_2, ceramide, tumor necrosis factor-α (TNF-α), ultraviolet radiation, cisplatin, and etoposide[22,25-27]. In fact, the *PKCδ* gene is deleted in many cancers[28]. Ectopic expression of PKCδ has been shown to decrease the anchorage-independent growth of NIH3T3 cells and reverse the transformation of rat fibroblasts and colonic epithelial cells by Src. Low levels of PKCδ have been reported in colon cancer, and overexpression of PKCδ suppresses the neoplastic phenotype of colon cancer cells[25]. A recent report suggested that PKCδ is lost in human squamous cell carcinoma due to transcriptional repression[29]. PKCδ has also been reported to decrease cell migration in breast cancer cells, whereas knockout of the PKCδ gene increases cell migration in mouse embryonic fibroblasts. These studies strongly support the role of PKCδ in tumor suppression.

Multiple reports suggest that PKCδ is responsible for apoptotic signaling in liver cancer cells (Table 1). In sorafenib-resistant hepatocellular carcinoma (HCC) cells, PKCδ activation was shown to induce cellular apoptosis *via* p38 activation[30]. Annexin A3 (ANXA3) interacts with PKCδ and thereafter suppresses PKCδ/p38-associated apoptosis and activates autophagy for cell survival. Thus, inhibition of ANXA3 by a monoclonal antibody is likely to impair cell survival and tumor growth. Although FTY720, a synthetic sphingosine immunosuppressor, has been known to have antitumor effects on HCC cells, PKCδ activation occurs in FYT720-treated HCC cells. FTY720 is thought to activate PKCδ *via* the generation of reactive oxygen species (ROS) and subsequent caspase 3-dependent cleavage to induce apoptosis. The relationship between intracellular activation of PKCδ and apoptosis in HCC cells has also been reported in the antitumor mechanism of an antagonist of FZD7, which is a membrane receptor overexpressed in HCC[31]. These lines of evidence suggest that PKCδ activation is not favorable for malignant transformation in liver cancer and may

Table 1 The relationship between subcellular localizations and functions of protein kinase Cδ in liver cancer

Response	Localization	Function	Mechanisms	Ref.
ANXA3 expression	Cytosol/plasma membrane	Interacts with PKCδ and inhibits apoptosis	p38MAPK activation	[30]
ROS	Nucleus	Activates PKCδ and induces apoptosis	Activates caspase 3 and induces cleavage of PKCδ	[31]
Claudin-1	Cytosol/plasma membrane	Enhances the ability of cell migration/invasion	Induces c-Abl-PKCδ signaling	[33]
mtROS	Plasma membrane	Induces gene expression for cell migration	Triggers oxidation of HSP60 and then induces MAPK activation	[34]
HIF-2α expression	Cytosol/plasma membrane	Induces cell migration	Phosphorylates PKCδ at Tyr311	[35]
HSP27 expression	Cytosol/plasma membrane	Inversely correlates with tumor malignancy	p38MAPK activation by PKCδ induces phosphorylation of HSP27	[36]
No response	Extracellular space/cell surface	Enhances cell proliferation	Activates MAPK signaling	[9]

mtROS: Mitochondrial reactive oxygen species; ANXA3: Annexin A3; PKCδ: Protein kinase Cδ; MAPK: Mitogen-activated protein kinase; HIF: Hypoxia-inducible factor; HSP: Heat shock protein.

be inactivated in these cells.

Tumor promotive function

Many studies have shown that PKCδ promotes the survival of multiple types of cancers, including non-small cell lung cancer, breast cancer, pancreatic cancer, chronic lymphocytic leukemia, and liver cancer.

PKCδ has been reported to induce signal survival. In fact, PKCδ promotes cell survival *via* several well-known pro-survival pathways, including NF-κB, Akt, and extracellular signal-regulated kinase (ERK). It has been reported that PKCδ inhibits apoptosis by inhibiting apoptosis protein-2 and FLICE-like inhibitory protein *via* NF-κB[32].

Numerous publications have reported that PKCδ is actively involved in the promotion of liver cancer, including cell migration, invasion, and tumor stage (Table 1). For example, claudin-1, a member of the tetraspanin family, plays a critical role in the acquisition of invasive capacity in human liver cells, and c-Abl-PKCδ signaling is important for malignant progression induced by claudin-1[33]. This c-Abl-PKCδ signaling pathway was shown to activate MMP-2, a key factor in cell migration and invasion. The cross talk between PKC and ROS may induce mitogen-activated protein kinase (MAPK) activation for cell migration and progression. Mandal *et al*[34] found that activation of PKCδ generated mitochondrial ROS triggers the oxidation of heat shock protein 60 (HSP60), a chaperone protein in the mitochondria, which induces the activation of ERK and c-Jun N-terminal kinase (JNK) in the cytosol, resulting in gene expression leading to migration in liver cancer. PKCδ and hypoxia have also been reported to be associated with cell migration in liver cancer. Hypoxia-inducible factor-2α expression regulates CUB domain-containing protein 1, which stimulates the phosphorylation of PKCδ at Tyr311 to induce malignant migration in various cancer cells, such as liver cancer cells[35]. Furthermore, the levels of HSP27 are inversely correlated with tumor stage, as per the tumor, node and metastasis classification, in patients with HCC. Takai *et al*[36] showed that PKCδ activation regulates the phosphorylation of HSP27 *via* p38 MAPK.

There is supportive evidence that PKCδ acts as a tumor promoter in many types of cancers. For example, the mRNA levels of PKCδ were higher in estrogen receptor (ER)-positive tumors than in ER-negative tumors, and an increase in PKCδ mRNA was associated with reduced overall survival[37]. PKCδ knockdown decreased the survival of MCF-7 and MDA-MB-231 breast cancer cells[38]. Overexpression of PKCδ was also observed in human ductal pancreatic carcinomas compared to its normal counterparts. PKCδ has been reported to be associated with melanoma cell metastasis[39]. A recent study demonstrated that integrin αvβ3-mediated invasion of melanoma cells is mediated *via* PKCα and PKCδ.

SUBCELLULAR LOCALIZATIONS AND FUNCTIONS OF PKCδ

Cytosol and plasma membrane

PKCδ is translated on the ribosome in the cytosol and generates its inactive cytosolic form. Similar to other PKC families, in response to DAG, PKCδ is also translocated to the plasma membrane *via* the C1 domain, which exerts a subsequent phosphorylation response. PKCδ activation is also required for Akt activation by Ras[40] (1-98). Activating mutations with Ras or PI3K increases PKCδ levels and induces Akt activation. PKCδ also induces ERK1/2 activation[41,42]. Akt and ERK1/2 activation have been implicated in the PKCδ-mediated increase in anchorage-independent growth and resistance of pancreatic ductal cancer cells to apoptotic stimuli[43]. Conversely, cytosolic PKCδ reportedly triggers apoptosis by activating p38 MAPK to inhibit Akt[8], indicating that PKCδ activation can behave as both a prosurvival and pro-apoptotic factor. Liver damage has been reported to induce inflammation and PKCδ translocation to the plasma membrane[44,45]. PKCδ activation has been observed in the tissues of patients with non-alcoholic steatohepatitis and non-alcoholic fatty liver disease and in a mouse model of hepatic cirrhosis[46-49].

Nucleus

Importantly, PKCδ is a PKC isoform that has been identified as a substrate for caspase-3[50]. Cleavage of PKCδ by caspase-3 separates the regulatory domain and catalytic fragment to allow constitutive activation of PKCδ even in the absence of any co-factors [22] and then translocates to the nucleus, where the catalytic fragment of PKCδ induces apoptosis[22]. Others and we have shown that full-length or fragmented PKCδ is translocated to the nucleus by transiting the nuclear pore[6,51]. Nuclear PKCδ interacts with and phosphorylates its substrates such as α-Abl, p53, p73, lamin B, Rad9, topoisomerase II, heterogeneous nuclear ribonucleoprotein K (hnRNP-K), and DNA-dependent protein kinase[22,52-54]. Moreover, nuclear PKCδ regulates the transcription of target genes in response to cellular stresses such as DNA damage, which is implicated in pro-apoptotic functions.

Upon oxidative stress, we previously showed that PKCδ associates with and activates IKKα in the nucleus[55]. Although IKKα activates NF-κB by phosphorylating IκB in the cytoplasm, which leads to prosurvival signaling, PKCδ-mediated IKKα activation at the nucleus causes phosphorylation of p53 at Ser20; however, it does not affect NF-κB activation.

The tumor suppressor p53 is a master regulator of cellular processes, such as cell cycle arrest, DNA repair, or apoptosis[56,57]. Several studies have suggested that p53 is located downstream of PKCδ. In response to genotoxic stress, PKCδ phosphorylates p53 at Ser46 to trigger p53-mediated apoptosis.

In the nucleus, PKCδ also regulates p53 expression by increasing *p53* transcription. We previously reported that PKCδ interacts with the death-promoting transcription factor Btf to induce Btf-mediated *p53* gene transcription and apoptosis[58]. In addition, TNF-α treatment induces translocation of PKCδ into the nucleus[59]. PKCδ can bind to the NF-κB RelA subunit and subsequently induce the transactivation of p65/RelA[59]. These findings demonstrate that NF-κB is involved in PKCδ-mediated TNF/TNF-related apoptosis-inducing ligand (TRAIL) resistance. PKCδ inhibition or knockdown decreased NF-κB expression and sensitized MCF7 cells to TNF/TRAIL-induced cell death[60].

Mitochondria

Bax and Bak are pro-apoptotic factors, and the Bcl-2 family regulates mitochondrial membrane permeability to induce apoptosis[61]. Upon exposure to ionizing radiation, Bax and Bak are activated *via* the c-Abl-PKCδ-p38 pathway to trigger mitochondrial cell death[30,62]. Mcl-1, an anti-apoptotic Bcl-2 family member, is a direct target of PKCδ. The catalytic fragment of PKCδ phosphorylates Mcl-1 and degrades it, leading to cell death. During the early stages of hypoxic stress, PKCδ induces autophagy *via* JNK-mediated phosphorylation of Bcl-2 to dissociate the Bcl-2/beclin-1 complex, and prolonged hypoxic stress induces PKCδ cleavage[63].

Cell surface

We recently showed that PKCδ is localized at the cell surface of liver cancer cell lines (Figure 2). Cell surface PKCδ was found to be anchored by other cell surface proteins, such as heparan sulfate proteoglycans (HSPGs). Some growth factors, such as fibroblast growth factors, vascular endothelial growth factor, and hepatocyte growth factor[64,65] have cationic amino acid clusters that can interact with heparan sulfate,

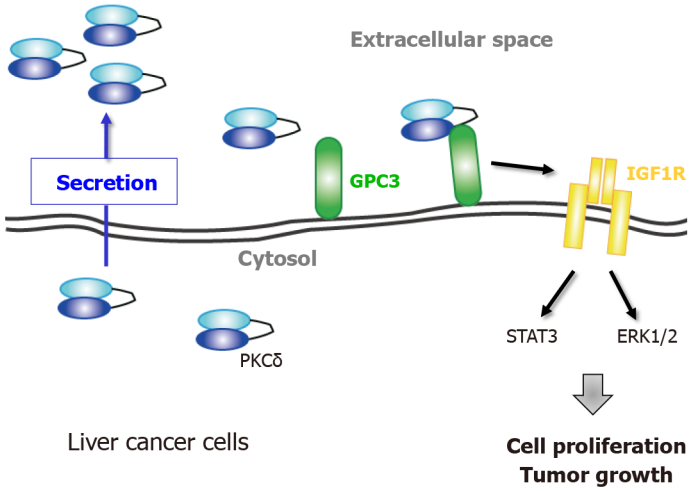


Figure 2 Extracellular protein kinase Cδ shows oncogenic property in liver cancer. Model of the proliferative regulation of extracellular protein kinase Cδ (PKCδ) in liver cancer cells. PKCδ is secreted from living cells and resides at the plasma membrane through its association with glypican 3, leading to an increase in insulin-like growth factor 1 receptor activation and enhancement of subsequent proliferative signaling to increase cell growth. PKCδ: Protein kinase Cδ; GPC3: Glypican 3; ERK: Extracellular signal-regulated kinase; IGF1R: Insulin-like growth factor 1 receptor.

which is composed of one or more unbranched anionic polysaccharide(s) known as glycosaminoglycans[65-67]. The cationic amino acid clusters closely resemble the NLS of intracellular proteins[68]. In fact, extracellular NLS-containing proteins, such as importin α1, huRNP-K, and PKCδ are detected at the cell surface of human cells[9,69,70]. These extracellular NLS-containing proteins are more likely to be located at the cell surface by binding to HSPGs.

Furthermore, glypican3, a liver cancer-specific HSPG, was identified as a receptor for cell surface PKCδ[71]. Both Cheng *et al*[72,73] and we showed that GPC3 regulates the activation of insulin-like growth factor 1 receptor (IGF1R)[9]. In fact, we found that extracellular PKCδ induces activation of IGF1R *via* association with GPC3 and its downstream signaling molecules, such as ERK1/2 and STAT3. Thus, these lines of evidence strongly suggest that cell surface PKCδ acts as a growth factor. In addition, we showed that anti-PKCδ monoclonal antibody (mAb) inhibits the proliferation and tumorigenesis of liver cancer cells, but not PKCδ-CRISPR knockout cells. Thus, cell surface PKCδ may be a potential therapeutic target for liver cancer.

Extracellular space

We also found that PKCδ is secreted into the extracellular space in liver cancer[9]. Extracellular accumulation of PKCδ was detected in different liver cancer cell lines but not in hepatocytes, suggesting that PKCδ secretion may be specific to liver cancer cells. Interestingly, our proteomics study showed that PKC, rather than PKCδ, was not detected in the culture medium of liver cancer cell lines. This means that PKCδ is a unique isoform of the PKC superfamily that is secreted extracellularly. Furthermore, higher levels of PKCδ were detected in the serum of patients with liver cancer, but not in patients with chronic hepatitis, hepatic cirrhosis, or healthy donors. This increase in serum PKCδ levels was also noted in a limited number of AFP- and PIVKA-II-negative liver cancer patients. Based on these clinical data, we propose that serum PKCδ may be a novel biomarker for liver cancer.

Recently, we and other groups have reported the extracellular localization of proteins with no signal peptide-containing proteins, such as FGF1, FGF2, HMGB1, hnRNP-K, importin α1, and IL-1β[69,70,74-77]. Secretion of these proteins is referred to as unconventional secretion[74,78]. Since the *PKCδ* gene does not encode a signal peptide, the extracellular secretion of PKCδ is also categorized as unconventional secretion. PKCδ has been shown to be full-length in the extracellular space and continues to be released from growing cells[9]. Many studies have reported that IL-1β secretion often occurs in immune cells after induction of inflammatory stimulation[77,79,80]. There are some differences in the secretion modes between immune and cancer cells. Unlike immune cells (IL-1β), liver cancer cells constitutively secrete importin α1 and PKCδ even under physiological culture conditions (using 10% FBS medium)[9,69]. Conversely, some features were common between immune and liver cells, including the induction of unconventional secretion by ATP treatment[81] and independent of brefeldin A, an inhibitor in the "conventional" secretion pathway of

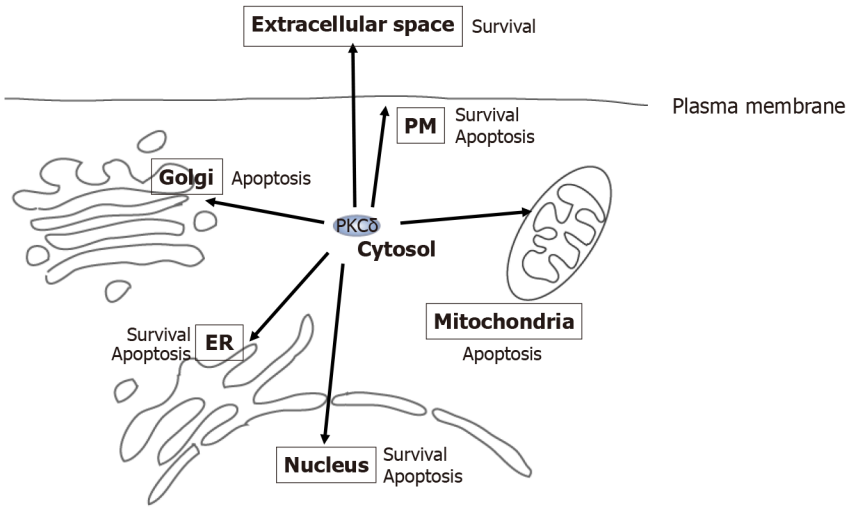


Figure 3 Multi-localization and functional diversity of protein kinase Cδ. Protein kinase Cδ (PKCδ) resides at various locations, including the cytosol, nucleus, estrogen receptor (ER), mitochondria, Golgi, extracellular space, and plasma membrane (inside and outside the cell). At each location, PKCδ acts as an apoptotic or survival factor in response to various stimuli, such as genotoxic stresses, phorbol ester, DNA damage, ER stress, tumor necrosis factor (TNF)-α, and TNF-related apoptosis-inducing ligand. PKCδ: Protein kinase Cδ; ER: Estrogen receptor; PM: Plasma membrane.

signal peptide-containing proteins[82].

We found that PKCδ secretion was initiated in the cytosol. Phorbol ester treatment inhibited PKCδ secretion, and the NLS active mutant was not secreted into the extracellular space. In fact, secreted PKCδ showed a lower level of phosphorylation (Tyr311 and Thr505). These lines of evidence support the possibility that cytosolic PKCδ, as a starting point for extracellular localization, could contribute to tumor progression in liver cancer.

Other organelles

A previous study has shown that PKCδ is translocated to the ER in response to ER stress and interacts with ER-bound c-Abl[83]. This PKCδ-c-Abl complex consequently moves to the mitochondria to trigger apoptosis[83]. It has been reported that tyrosine phosphorylation of PKCδ is associated with this interaction with c-Abl[84]. The chemical inhibitor rottlerin blocks the translocation of the PKCδ-c-Abl complex from the ER to the mitochondria, which confers protection against apoptosis[83]. Another ER protein, p23 (Tmp21), interacts with PKCδ, which enables the retention of PKCδ in the ER[85]. Translocation of PKCδ to the ER has also been reported in cells with Sindbis virus and in glioma cells treated with TRAIL, where PKCδ exerts an anti-apoptotic effect. Furthermore, a small amount of PKCδ has been observed in the Golgi apparatus. Ceramide or IFN-γ stimulation has been shown to translocate PKCδ to the Golgi apparatus, which is associated with ceramide-induced apoptosis in HeLa cells.

CONCLUSION

The apoptotic and survival functions of PKCδ are defined by cell and tissue types and their cellular conditions (Figure 3). In response to cellular stresses, PKCδ may be translocated to different organelles (including the cytosol and extracellular space), where PKCδ executes distinct functions in each location. Among the many types of tissues and cells, liver cancer cells have the most patterns of localization of PKCδ, including conventional intracellular and extracellular localization. Notably, extracellular PKCδ is involved in the tumorigenesis of liver cancer; therefore, it is a promising novel diagnostic and therapeutic target for liver cancer. Additional studies are required to elucidate further the various roles of PKCδ in liver cancer cells, which are dependent on the expression, subcellular distribution, and tumor microenvironment.

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MINIREVIEWS

Therapeutic endoscopy for the treatment of post-bariatric surgery complications

Michael Larsen, Richard Kozarek

ORCID number: Michael Larsen 0000-0001-8005-0750; Richard Kozarek 0000-0002-9745-7538.

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Michael Larsen, Richard Kozarek, Digestive Disease Institute, Virginia Mason Medical Center, Seattle, WA 98101, United States

Corresponding author: Michael Larsen, MD, Doctor, Digestive Disease Institute, Virginia Mason Medical Center, 1100 Ninth Avenue, Seattle, WA 98101, United States.
michael.larsen@virginiamason.org

Abstract

Obesity rates continue to climb worldwide. Obesity often contributes to other comorbidities such as type 2 diabetes, hypertension, heart disease and is a known risk factor for many malignancies. Bariatric surgeries are by far the most invasive treatment options available but are often the most effective and can result in profound, durable weight loss with improvement in or resolution of weight associated comorbidities. Currently performed bariatric surgeries include Roux-en-Y gastric bypass, sleeve gastrectomy, and laparoscopic gastric banding. These surgeries are associated with significant weight loss, but also with significant rates of major complications. The complexity of these patients and surgical anatomies makes management of these complications by a multidisciplinary team critical for optimal outcomes. Minimally invasive treatments for complications are typically preferred because of the high risk associated with repeat operations. Endoscopy plays a large role in both the diagnosis and the management of complications. Endoscopy can provide therapeutic interventions for many bariatric surgical complications including anastomotic strictures, anastomotic leaks, choledocholithiasis, sleeve stenosis, weight regain, and eroded bands. Endoscopists should be familiar with the various surgical anatomies as well as the various therapeutic options available. This review article serves to delineate the current role of endoscopy in the management of complications after bariatric surgery.

Key Words: Therapeutic endoscopy; Bariatric surgery; Complications; Weight regain; Sleeve stenosis; Sleeve leak

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Core Tip: Bariatric surgery is the most effective treatment for morbid obesity. While surgical techniques have improved, complications after these surgeries remain

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common. Multidisciplinary management of these complications is important given their complexity. Therapeutic endoscopy provides a minimally invasive option for treatment of complications. This review article serves to delineate the current role of therapeutic endoscopy in the management of complications after bariatric surgery.

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INTRODUCTION

Obesity rates have grown dramatically both in wealthy nations and the developing world; resulting in a worldwide global health problem. According to the WHO, in 2016, 1.9 billion people worldwide were affected by this disease[1]. Obesity often contributes to other comorbidities such as type 2 diabetes, hypertension, heart disease and is a known risk factor for many malignancies[2]. Obesity can dramatically affect a patient's quality of life and significantly reduces life expectancy.

Many treatments for obesity exist including dieting, exercise, weight loss medications, endoscopic bariatric therapies, and bariatric surgery[3]. However, treatment is difficult as most therapies result in, at most, moderate weight loss and once the therapy is withdrawn, significant weight recidivism occurs[4]. Bariatric surgeries are by far the most invasive treatment options available but are often the most effective and can result in profound, durable weight loss with improvement in or resolution of weight associated comorbidities[5]. For this reason, 256000 people underwent bariatric surgery in the United States in 2019, and 696191 underwent surgery worldwide in 2018[6,7]. While bariatric surgery is very effective, it does represent a major operation often being performed in high-risk individuals with multiple comorbidities. While surgical techniques have improved, and these surgeries have become safer over time, they remain associated with significant complications. About 9%-12% of patients undergoing bariatric surgery will experience 1 or more adverse events in the first five years after surgery[8]. While most complications will be minor, they can also be life threatening or associated with significant morbidity. These are often very complex patients such that adverse events are best managed by a multidisciplinary bariatric team which can include bariatric surgeons, endocrinologists, interventional radiologists, dieticians, and gastroenterologists. Minimally invasive, endoscopic or percutaneous treatments are preferred as reoperation is associated with significant additional risk. This review will focus on the large subset of adverse events after bariatric surgery that can be managed through endoscopic techniques (Table 1).

BARIATRIC SURGERY

Effective endoscopic treatment of post-bariatric surgery complications requires a detailed understanding of the gastrointestinal anatomy created by the particular surgical procedure. Currently the most performed surgical procedures are the sleeve gastrectomy and the laparoscopic Roux-en-Y gastric bypass (RYGB). Less commonly performed surgeries include laparoscopic adjustable gastric banding (LAGB) and the duodenal switch. Gastroenterologists will also still encounter patients with complications after vertical banded gastroplasty though this procedure is no longer performed.

RYGB

Despite the fact that it has lost some degree of popularity secondary to the rise of the sleeve gastrectomy, Roux-en-Y gastric bypass remains a commonly chosen bariatric surgery because of its excellent efficacy. Originally an open surgical procedure, gastric

Table 1 Endoscopic management of bariatric complications

Surgery	Complication	Diagnosis	Management options
RYGB	Gastrojejunal anastomotic stricture	Upper GI series; Endoscopy	Endoscopic balloon dilation; Steroid injection; Needle knife radial incisions; Lumen-opposing metal stent
	Gastrogastric fistula	Upper GI series; Endoscopy	Endoscopic suturing; OTSC
	Anastomotic leaks	CT imaging; Upper GI series; Endoscopy	CSEMS; Internal drainage with pigtail stents; OTSC; Endosponge therapy; Endoscopic suturing
	Choledocholithiasis	MRCP; CT imaging; Ultrasound	Overtube-assisted ERCP; Laparoscopic-assisted ERCP; EDGE
	Weight regain	EGD-Dilated gastrojejunostomy	Stoma reduction: Endoscopic suturing; OTSC; Serial APC treatments; Radiofrequency ablation
Sleeve gastrectomy	Staple line leak	CT imaging; Upper GI series; Endoscopy	CSEMS; Internal drainage with pigtail stents; OTSC; Endosponge therapy; Endoscopic suturing
	Sleeve stenosis	Upper GI series; Endoscopy	Radial expanding balloon dilation; Pneumatic balloon dilation
LAGB	Band migration	CT imaging; Endoscopy	Mechanical lithotripter band cutting

RYGB: Roux-en-Y gastric bypass; CSEMS: Covered self-expandable metallic stent; OTSC: Over-the-scope-clip; EDGE: Endoscopic ultrasound-directed transgastric endoscopic retrograde cholangiopancreatography; APC: Argon plasma coagulation; LAGB: Laparoscopic adjustable gastric band.

bypass is now done nearly exclusively laparoscopically. This minimally invasive approach combined with improved surgical techniques have led to decreased morbidity. This surgery involves partitioning a small gastric pouch from the proximal stomach and the creation of a Roux limb which diverts most absorption to the distal small bowel. Weight loss occurs secondary to the restrictive effect of the small gastric pouch, malabsorptive effects, and complex hormonal changes that occur as a result of the bypass. Hormonal changes are likely responsible for the profound effect this surgery has not just on the treatment of obesity but also for diabetes[9]. Surgical anatomies are not uniform and highly operator dependent. Details such as the size of the gastric pouch, diameter of the gastrojejunostomy, length of the Roux limb, and length of the pancreatobiliary limb will vary significantly by surgeon.

Complications of this surgery can manifest at any point after surgery with GI bleeding and leaks typically presenting early while vitamin deficiencies may take years to manifest. Possible adverse events related to this surgery include marginal ulceration, anastomotic strictures, gastrogastric fistulas, anastomotic leaks, choledocholithiasis, dumping syndrome, metabolic abnormalities, vitamin deficiencies, or chronic abdominal pain among others.

Anastomotic strictures

Stricturing at the gastrojejunal anastomosis is a common adverse event after RYGB which can occur as early as the first few weeks post-operatively or many years after the surgery. In some surgical series, the frequency of strictures is in the 20%-30% range [10]. The exact etiology of these strictures has not been completely elucidated-ulceration, ischemia, and minor anastomotic leaks as well as foreign body reactions to staples or suture are likely contributors. Surgical technique likely contributes as it has been shown that operations involving circular staplers are more likely to stricture[11].

Patients with anastomotic strictures typically present with nausea and vomiting symptoms or in the early post-operative period will report inability to advance their diet beyond liquids. Some stricture patients will describe dysphagia while others may endorse significant post-prandial abdominal pain. Symptoms of gastrojejunal stricturing are often very similar to those of marginal ulceration and differentiating between these 2 adverse events prior to endoscopic evaluation can be very difficult. Tight strictures can be diagnosed using a radiologic upper GI series, but more subtle narrowing can be missed as liquid will continue to pass through to the jejunum. Some patients with strictures will have inadvertent weight gain as they will compensate for the stricture by sticking to a high calorie liquid diet.

All patients with symptoms that are suggestive of a possible anastomotic stricture should undergo an upper endoscopy for diagnosis and treatment. Normal anastomotic diameter should be between 10 mm and 15 mm, therefore a stricture can be diagnosed in any patient in which the standard upper endoscope cannot pass easily through the

anastomosis into the jejunum. Treatment for the stricture can then be performed immediately after diagnosis through endoscopic through-the-scope balloon dilation. Fluoroscopy can often be helpful to ensure the correct positioning of the balloon and to avoid trauma to the thin jejunum. Therapy should be aimed at dilating the stricture to achieve a luminal diameter of 10-15 mm. Dilation should be gradual and often requires more than 1 dilation session-particularly in patients with very tight strictures. Care should be taken to avoid over dilating the anastomosis as this is associated with an increased risk of complications and can result in the patient experiencing a decreased sense of restriction and ultimately result in weight gain. Overall, endoscopic balloon dilation is safe, with very few complications and is effective for most patients[12,13] (Figure 1).

Multiple options are available for the treatment of refractory strictures. Increasing balloon diameter combined with intralesional steroid injection can be effective. Our group will typically utilize Triamcinolone 10 mg/mL with 1-2 mL injected in a 4-quadrant fashion. An additional technique which can be added is incisional ablation of the stricture with radial cuts with a needle knife prior to dilation therapy. For patients who continue to be refractory, these strictures can be treated with endoscopic stenting. While treatment with esophageal stents in gastric bypass patients is typically poorly tolerated secondary to associated side-effects (pain, nausea, vomiting, reflux) and frequent stent migration, treatment with new lumen apposing metal stents (LAMS) is well tolerated and effective[14,15]. Our group has had good success with the 10 mm × 15 mm LAMS but in patients whose strictures continue to recur, the 20 mm stent may also be effective. Truly refractory strictures which require a surgical revision are very rare and usually represent narrowing secondary to twisting/torsion of the post-anastomotic jejunum rather than true fibrotic strictures of the anastomosis.

Gastrogastric fistula

A gastrogastric fistula is a communication between the gastric pouch and the gastric remnant. In the traditional open RYGB surgery this was a common occurrence seen in up to 30% of patients. However, in laparoscopic patients this is a much less frequently seen complication secondary to changes in surgical technique[16]. In open surgeries the gastric pouch was partitioned using a staple line but was not completely separated from the defunctionalized stomach as is done in laparoscopic RYGB. While the exact etiology of these fistulas is not known, anastomotic leaks, ulceration, ischemia, and erosions of foreign bodies are thought to contribute[17-19]. The presenting symptoms of gastrogastric fistula patients are highly variable and can range from being completely asymptomatic to refractory or perforating marginal ulcers. Weight regain is a common occurrence with these fistulas as they can allow a significant amount of food to pass into the gastric remnant which functionally reverses the effects of the surgery. Gastric acid can pass through the fistula from the gastric remnant to the pouch and result in gastroesophageal reflux disease, abdominal pain, marginal ulcers, and anastomotic strictures.

Gastrogastric fistulas can be diagnosed *via* upper endoscopy, upper GI series, or sometimes *via* computed tomography (CT) scan. While the radiological tests can be helpful one must be careful not to overinterpret the presence of contrast in the gastric remnant as contrast can often reflux up the pancreatobiliary limb. Endoscopic diagnosis is particularly helpful as it provides the most accurate measure of the size of the fistula which is important in determining treatment.

Patients with symptoms of GERD or other consequences of gastric acid passage through the gastrogastric fistula can often be treated with therapy with proton pump inhibitors or other antacids alone. Indications for closure of a gastrogastric fistula include refractory symptoms or significant weight regain.

Endoscopic therapy is most effective for smaller fistulas-particularly those that are smaller than 1 cm in diameter. Unfortunately, even these smaller fistulas have high rates of reopening after endoscopic closure[20]. The most studied endoscopic therapy is endoscopic suturing which results in a very high initial closure rate, but long-term efficacy may be as low as 20%[21]. The over-the-scope-clip (OTSC) is another option for fistula closure though it has been less studied for this indication[22]. In our experience these clips can be effective for small fistulas and can be used to reinforce an endoscopic suturing closure. One concern about using this device is that the large clip can interfere with surgical revision if that becomes necessary secondary to failure of closure. However, endoscopists now have access to a device specifically designed to help remove these large clips which can be done prior to a surgical closure[23]. Patients with persistent symptoms despite medical therapy, with large fistulas or those that are refractory to endoscopic closure should be evaluated by a bariatric surgeon for surgical revision.

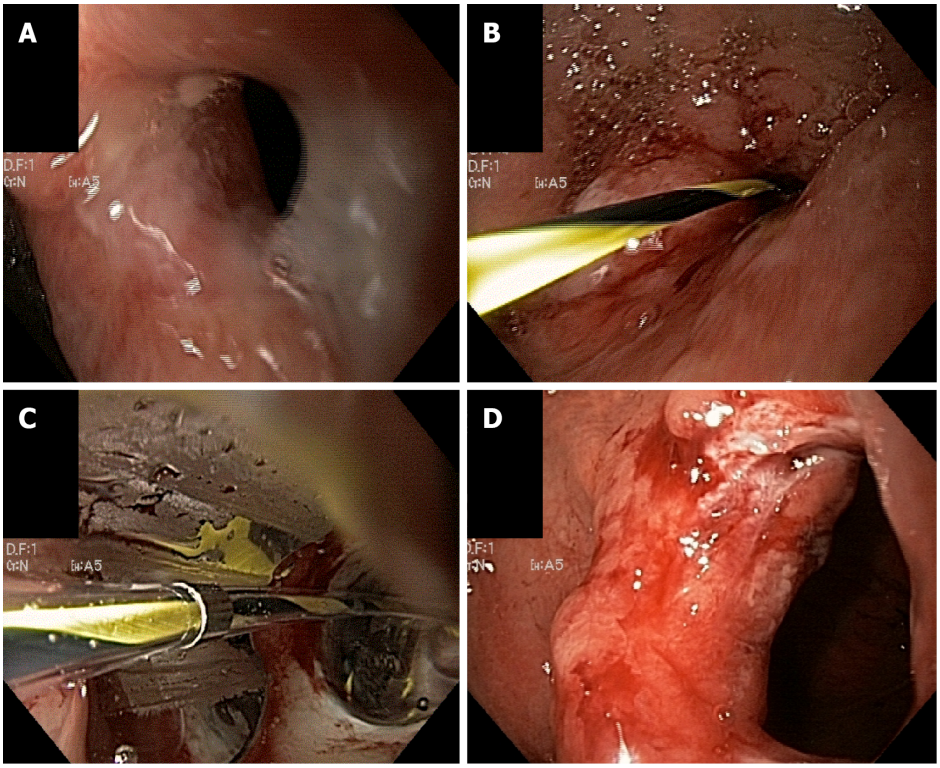


Figure 1 Anastomotic stricture dilation. A: Tight stricture of gastrojejunostomy; B: Wire placement through stenosis; C: Balloon dilation; D: Stricture appearance after dilation.

Anastomotic leaks

Anastomotic leaks can be the most severe and debilitating complications after bariatric surgery and are seen most commonly after sleeve gastrectomy (discussed in sleeve gastrectomy section) but can also be seen typically as a very early complication after RYGB[24]. RYGB involves the creation of multiple different surgical anastomoses, and leaks can occur at any of these sites including the gastric pouch, gastrojejunostomy, blind limb of jejunum, jejunojejunostomy, and the gastric remnant. Leak patients will typically present with abdominal pain, fevers, and potentially sepsis and clinical instability[25]. The initial treatment for all leak patients involves antibiotics, fluid resuscitation, and NPO status[26]. Patients who are clinically unstable will typically need to be taken to the operating room for a wash-out and attempted surgical closure. In more stable patients cross-sectional imaging should be obtained in order to evaluate the location of the leak.

Patients with leaks originating from the gastric pouch or gastrojejunostomy can be treated successfully with endoscopic therapy using covered endoluminal stents[27, 28]. The majority of published research on this treatment involves the use of self-expandable metal esophageal stents which can be fully covered or partially covered with plastic. Unfortunately, these stents are poorly tolerated by patients but can typically be removed in 6 to 8 wk with a leak resolution rate of 87.8%[29]. For leaks at the gastrojejunostomy, the much shorter, dumbbell shaped LAMS stents can be used and are significantly better tolerated, but currently experience with this technique is limited.

Another option for treatment of leaks is internal drainage, which involves the placement of double pigtail stents through the opening of the leak into the associated abscess cavity[30]. This drainage allows the leak to heal around the stents similar to what occurs in drainage of pancreatic fluid collections. The stents are then exchanged every 6 wk until resolution of the abscess cavity and leak are seen. This technique is much better tolerated than esophageal stents and has the benefit of being able to be performed at leak sites throughout the RYGB anatomy-even those that require an enteroscope to reach. Other options for treating leaks include endoscopic suturing, endoscopic clipping, eVAC therapy (transnasal wound vac with endosponge), as well as over-the-scope clips[31]. However, the published data for their use remains very limited.

Choledocholithiasis

The development of cholelithiasis is common after bariatric surgery and particularly so after RYGB. Up to 36% of patients will have gallbladder stones 6 months after surgery, and a large portion of those patients have gallstone-related symptoms[32]. Rapid weight loss is believed to increase the cholesterol saturation of bile, and anatomic changes occurring during the surgery may affect gallbladder emptying, both of which promote.

stone formation. Studies have shown that 2%-7% of symptomatic patients will have choledocholithiasis which is best treated by endoscopic retrograde cholangiopancreatography (ERCP), a procedure that is technically very challenging for post RYGB patients as the major papilla cannot be reached with a standard duodenoscope[33]. Currently there are 3 endoscopic options for performing ERCP in the RYGB patient-overtube-assisted enteroscopy ERCP, lap-assisted transgastric ERCP, or an endoscopic ultrasound-directed transgastric ERCP (EDGE).

Enteroscopy ERCP can be performed with a single- or double-balloon enteroscope, or spirus assisted, or in patients with short Roux limbs, with a colonoscope. Published data suggest a 70% efficacy rate of this technique[34]. Limitations are primarily related to the lack of appropriate length and size of devices and lack of elevator on the scope. Long Roux limbs or pancreatobiliary limbs can also affect the likelihood of success. Use of a clear cap on the end of the enteroscope can improve visualization and positioning of the scope in the descending duodenum.

Laparoscopic-assisted ERCP requires taking the patient to the operating room where a surgeon will create a gastrostomy in order to facilitate percutaneous passage of a standard duodenoscope into the second portion of the duodenum[35]. This access allows the endoscopist the ability to perform ERCP with standard devices and techniques. This technique has been shown to be effective with a technical success rate of 97.9%, but adverse event rates are higher (19%) than that seen with overtube-assisted ERCP (6.5%)[36]. For patients who need multiple ERCPs a gastrostomy tube can be left in place to provide an access tract, however repeat ERCP can be challenging as the duodenoscope will not pass easily through a standard gastrostomy tube tract.

EDGE is a new and still somewhat controversial technique for performing ERCP which involves the creation of a gastrogastric fistula with EUS-guided deployment of an Axios (Boston Scientific, Marlborough, MA) LAMS[37] (Figure 2). The first step in this process is to identify the gastric remnant from the gastric pouch with a linear EUS scope. A 19-gauge needle is then advanced into the gastric remnant and used to infuse water into the gastric remnant to distend it. If not already in the left lateral position, moving the patient onto their left side can facilitate keeping this fluid in the gastric remnant. Once the gastric remnant is distended with fluid a LAMS can be deployed in a similar fashion to draining a pancreatic pseudocyst. As the gastric remnant is typically separated from the gastric remnant the stent does cross the serosa of the gastric pouch and the gastric remnant such that if the stent migrates a perforation will occur. Therefore, this procedure is done most safely if the stent is deployed and then 2-3 wk is allowed for a tract to form along the stent prior to passing the duodenoscope through to perform ERCP[38]. For those patients that cannot wait for their ERCP, the stent should be sutured or clipped in place, and the stent should not be removed immediately after the procedure. Once the LAMS stent is in place a duodenoscope can pass through it and then achieve standard positioning for ERCP. If repeat ERCPs are needed the stent can be left in place, but plans should be made to remove the stent as soon as possible to reduce the risk of a persistent gastrogastric fistula. Data on this technique is limited, though a retrospective multicenter review of 178 patients demonstrated a technical success of 98% and only 4 patients with adverse events[39]. It remains controversial because of the risk of persistent gastrogastric fistula which was 10% in this review.

ERCP for patients after RYGB is challenging and should involve a multidisciplinary team. Bariatric surgery consult should be considered prior to embarking on any of the 3 potential endoscopic treatment options. Balloon-assisted ERCP is our first line therapy for most ERCP indications, but a plan should be in place prior to the procedure as to what second-line option will be utilized in the event that this is not successful. If EDGE is planned after failed balloon-assisted ERCP, then water can be pumped quickly into the gastric remnant through the enteroscope prior to exchanging for the linear EUS scope, and the LAMS is deployed to create the gastrogastric fistula as part of the same procedure.

Weight regain

Patients who undergo Roux-en-Y gastric bypass typically experience a profound

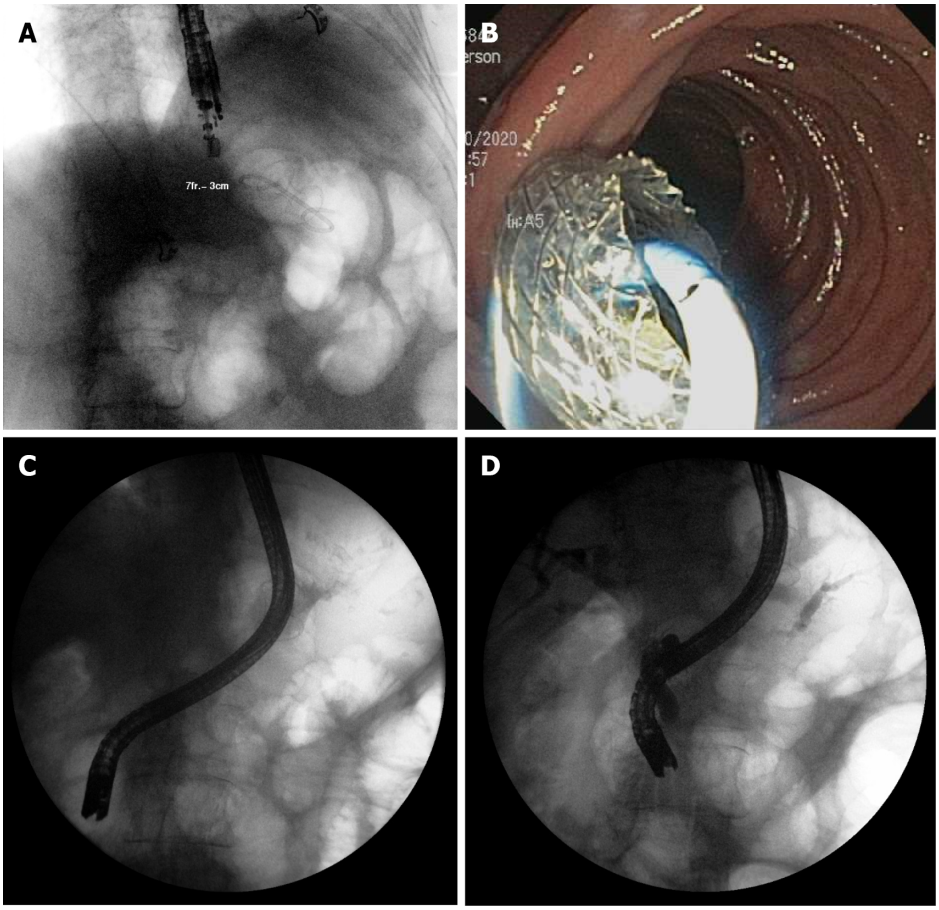


Figure 2 Endoscopic ultrasonography-directed transgastric endoscopic retrograde cholangiopancreatography. A: Endoscopic ultrasonography placement of lumen apposing metal stents (LAMS) gastrogastric fistula; B: Endoscopic view of LAMS; C: Duodenoscope passing through LAMS for endoscopic retrograde cholangiopancreatography; D: Successful cholangiogram and pancreatogram.

weight loss in the first 6 mo after surgery. While the rate of weight loss will slow after this initial period, significant weight loss will continue for the first 12-15 mo after which patients will typically hit a weight loss plateau with expected total weight loss of roughly 30% total weight or 60% excess weight loss. It is common for patients to gain back a small amount (10%) of this significant weight loss over the subsequent 1-2 years. However, about 18%-30% of RYGB patients will suffer from significant "weight regain"-a term which generally denotes regaining more than 50% of the initial weight loss[40-42]. This kind of weight gain can be devastating emotionally for these patients who have undertaken significant surgery only to see their health gains eliminated.

The etiology of weight regain after RYGB is often multifactorial and can involve dietary, hormonal, and behavioral factors but also often involves anatomic changes to the RYGB anatomy. Gastrogastric fistulas can contribute significantly to weight gain and their management was discussed earlier in this review. Dilation of the gastric pouch and/or dilation of the gastrojejunostomy are other anatomic changes that have been shown to be associated with weight gain in RYGB patients[43]. Presumably these changes result in weight gain by removing the sense of restriction or satiety that these patients are used to experiencing after eating. A dilated gastrojejunostomy will allow food to empty the gastric pouch very quickly after eating. These patients will often describe an initial sense of fullness after eating which passes very quickly, resulting in frequent hunger and resultant increase in caloric intake. While a gastrogastric fistula can be diagnosed by upper GI series these anatomical changes are typically best evaluated by endoscopy as an upper GI series cannot measure the size of the gastrojejunostomy.

Surgical revisions for alterations in gastric bypass anatomy are associated with very high rates of adverse events - making minimally invasive endoscopic treatments better options. The most studied endoscopic treatment for a dilated gastrojejunostomy is to narrow the stoma diameter using endoscopic suturing (Figure 3). This has been done with multiple different suturing platforms and has been shown to be effective for weight loss in a sham controlled randomized controlled trial[44]. Data with the newer

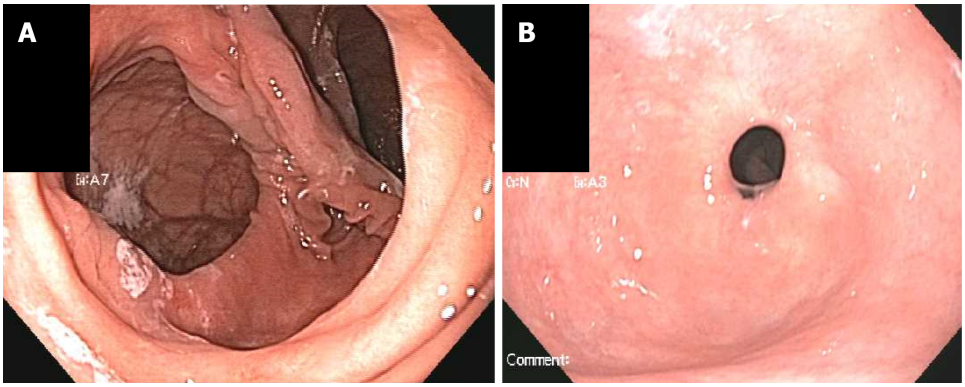


Figure 3 Dilated gastrojejunostomy treated with endoscopic suturing. A: Dilated gastrojejunostomy; B: Gastrojejunostomy several months after suturing for stoma reduction.

Overstitch (Apollo Endosurery, Austin Tx) device which allows full thickness suturing demonstrates that this device is the most effective[45]. Argon plasma coagulation (APC) treatment to the 2 cm of tissue on the gastric side of the gastrojejunostomy to expose the submucosa is typically performed prior to suturing. Multiple different suturing patterns have been used with some published data suggesting that a circumferential running stitch pattern provides a more robust weight loss than interrupted suture patterns[46]. The goal of suturing is typically to reduce the diameter of the gastrojejunostomy to 8 to 10 mm. When this is achieved, patients will typically stop regaining weight and data from the largest published study (n = 331) demonstrates weight loss of 8.5% ± 8.5% at 1 year.

Long-term follow-up has also shown persistent effect of the intervention with patients losing 8.8% ± 12.5% total weight at 5 years[47]. For those patients who are not able to undergo endoscopic suturing there are other endoscopic options to narrow the gastrojejunostomy though data on these alternate methods at this point is limited. Investigators have shown that placement of an OTSC at 1 edge of the gastrojejunostomy or 2 clips at opposite sides of the gastrojejunostomy can be effective at narrowing the stoma diameter[48]. Alternatively, serial sessions of treatment with APC alone to the rim of the gastrojejunostomy without any suturing can be effective [49]. In this technique the APC is applied with some degree of contact with the mucosa in order to achieve a deeper penetration of the thermal effect. This technique is less effective for gastrojejunostomies with a diameter greater than 30 mm. One case series also described the use of serial sessions of radiofrequency ablation to the edges of the gastrojejunostomy with 18.8% excess weight loss at 12 mo[50]. Serial sessions with injection of the sclerosant sodium morrhuate has also been shown to be effective, but unfortunately this product is no longer available for purchase for medical usages.

SLEEVE GASTRECTOMY

The creation of a gastric sleeve was initially just 1 part of the more complex and rarely performed duodenal switch operation. However, since becoming a stand-alone surgery, sleeve gastrectomy has gained popularity over the past 2 decades and is now the most commonly performed bariatric surgery in the U.S. Sleeve gastrectomy now accounts for greater than 50% of all bariatric surgery[51].

The creation of the gastric sleeve involves the surgical excision of a large portion of the greater curve of the stomach. The surgeon then closes the resulting defect with a long staple line. The resulting anatomy is a narrow tubular stomach. The surgery is thought to induce weight loss in multiple different ways. Resection of the greater curve of the stomach results in the removal of the majority of the ghrelin-producing cells which results in decreased hunger[52]. Additionally, the resultant tubular sleeve has much less capacity than a normal stomach and results in greater satiety after eating. Interestingly, the sleeve gastrectomy is thought to result in quicker gastric emptying which results in weight loss through various hormonal effects. Overall, the weight loss seen with this surgery is significant and can be similar to that seen with Roux-en-Y gastric bypass[53].

While typically considered a more straight-forward surgery than RYGB, complications occur with similar frequency with the sleeve gastrectomy[54]. Common complications which can occur and be treated with therapeutic endoscopy include staple line leaks as well as the development of a stenosis of the gastric sleeve.

Sleeve gastrectomy staple line leak

As discussed above with regard to leaks after RYGB, surgical leaks can occur at any surgical anastomosis. Leaks can be a devastating complication which can take a very long time and multiple endoscopic procedures to heal. The creation of a gastric sleeve results in a very long staple line anastomosis which extends from the antrum of the stomach up to the GE junction. Leaks are seen in up to 5% of sleeve gastrectomy patients and can occur anywhere along the staple line[55]. However, most leaks occur near the top of this staple line at the angle of His which is particularly susceptible to leaks because this area has a very thin gastric wall, often experiences relative ischemia secondary to surgical ligation of the short gastric arteries, has relative dysmotility, and is an area of increased intragastric pressure. Downstream obstructions such as a concomitant sleeve stenosis often contribute to the development of leaks and need to be addressed as part of treatment[56]. Sleeve leaks typically present in the early post-operative period, but chronic leaks can present at any point-sometimes even many years after their surgery.

Leak patients' clinical presentation typically varies based on the timing of their leak. Patients with acute leaks (< 7 d after surgery) typically present with signs of sepsis and are often hemodynamically unstable and require urgent operative intervention with abdominal wash-out and placement of abdominal drains. Patients with subacute or chronic leaks are typically less acutely ill and present with signs and symptoms including abdominal pain, fever, and tachycardia. Prompt evaluation is needed to reduce associated complications[57]. Diagnosis of a sleeve leak is typically made by a combination of CT scan and upper GI series. If a leak is suspected but not confirmed on upper GI series, then endoscopy with direct visualization can typically secure a diagnosis. Initial leak treatment involves initiation of broad-spectrum antibiotics, IV fluid resuscitation, NPO status, and surgical consultation.

While some leak patients will require re-operation, the majority can be treated with various endoscopic techniques. Unfortunately, this often involves a prolonged treatment course which can involve long hospital stays and significant impacts to quality of life. Many patients will require multiple different modalities of endoscopic treatment to achieve resolution of the leak[58]. Available endoscopic treatment options include covered esophageal stents, internal drainage, OTSC, endoscopic suturing, eVAC endosponge therapy, and endoscopic septotomy. Many patients will also require supplemental enteral nutrition delivered through a jejunostomy tube during their treatment.

The most commonly used endoscopic treatment for sleeve gastrectomy leaks is the use of covered self-expandable metal stents (CSEMS) (Figure 4). These stents treat sleeve leaks through 2 mechanisms-the stent both covers the leak orifice and treats the distal gastric stenosis and reshapes the stomach. Stents used can include both fully covered and partially covered stents. Our group utilizes more partially covered stents for this purpose because they result in decreased stent migration and can result in a more effective seal at the top of the stent as tissue grows into the partially covered portion of the stent. The downside to using partially covered stents is the increased difficulty of removing these stents once the leak has healed. In order to achieve successful removal of a partially covered stent one must place an overlapping fully covered stent within the stent. The expansile pressure from the second stent results in tissue necrosis of tissue ingrowth of the first stent and allows removal of both stents about 1 week after placement[59]. A recent systematic review and meta-analysis demonstrated that the use of CSEMS for treatment of sleeve leaks was effective in 72.8% of cases[60]. Unfortunately, use of these stents is associated with poor patient tolerance with symptoms of chest pain, gerd, nausea and vomiting, and frequent stent migration when fully covered stents are used. Because of the associated side effects of these stents, patients can rarely maintain adequate nutrition *via* PO intake, and therefore we typically place an endoscopic jejunal feeding tube at the initiation of treatment to provide supplemental enteral nutrition to promote leak healing. Multiple techniques have been tried to prevent stent migration including using hemoclips, over-the-scope-clips, and endoscopic suturing to attempt to secure the proximal aspect of the stent to the esophagus. However, migration can still occur despite these measures and was seen in 15.9% of patients in a large meta-analysis[61].

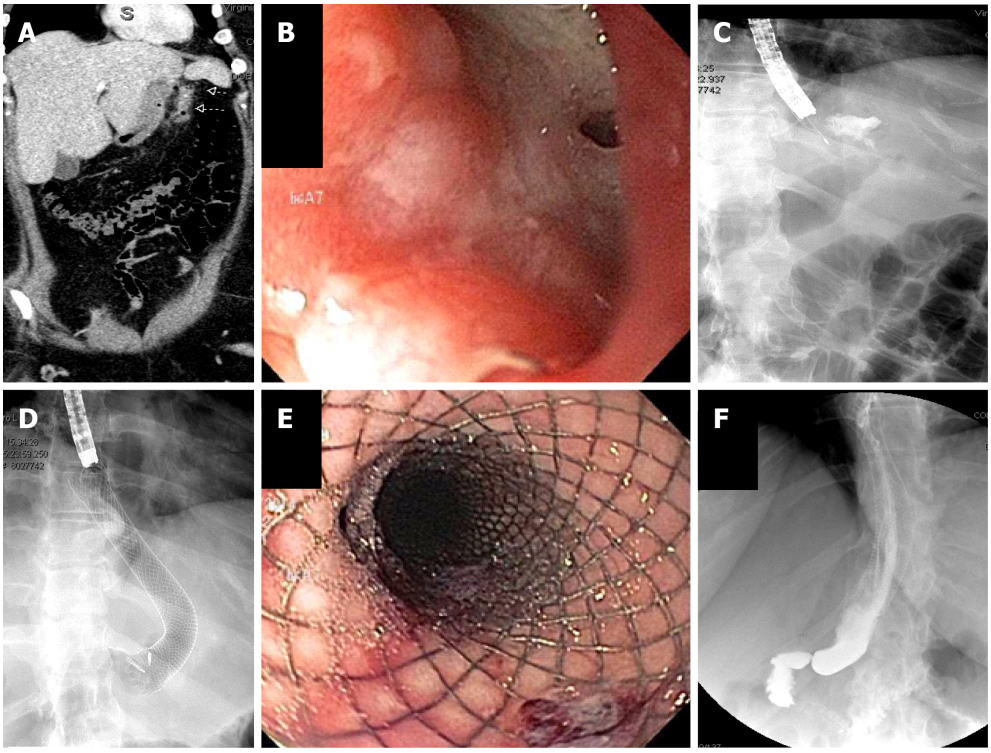


Figure 4 Sleeve leak treated with covered esophageal stent. Computed tomography imaging demonstrating sleeve leak; B: Endoscopic appearance of leak site; C: Contrast injection to confirm leak site; D: Placement of covered self-expandable metal stents; E: Endoscopic appearance of stent; F: Subsequent upper gastrointestinal series showing no residual leak after stent placement.

Internal drainage using transgastric double pigtail stents is a more recently described technique for treating sleeve gastrectomy leaks[30]. This involves the drainage of the associated abscess cavity with transgastric plastic stents (Figure 5). If the leak opening is large enough prior to stenting, the endoscope can be passed through the leak and used to lavage and debride the associated cavity prior to stenting. If there is a stenosis of the distal sleeve, then this should be combined with dilation of the sleeve as well. The stents are then exchanged every 6 wk until the cavity and leak have resolved through healing by secondary intention. Retrospective studies have shown improved decreased morbidity and mortality, and improved technical success when compared with the use of CSEMS. The largest published study included 67 sleeve leak patients and demonstrated a 72.8% clinical success rate and a mean of 3.18 endoscopic procedures[30].

In our experience, this technique is very well tolerated by patients, and PO intake can be started early which obviates the need for PEJ tube, and the internal drainage avoids the need for percutaneous drainage. This method of endoscopic treatment has become our primary method for treatment of sub-acute leaks, and some data has demonstrated effectiveness in acute leaks as well.

Other treatment options for leak closure include devices which attempt to directly close the leak orifice, including the use of through the scope clips, OTSC, and endoscopic suturing. Data on the use of these techniques in sleeve leaks is limited though retrospective case series, have shown that OTSCs and endoscopic suturing can be effective in some leak patients[62]. In our experience the typical location of sleeve leaks at the proximal end of the staple line makes access for suturing or clipping challenging. Furthermore, the leak site tissue is generally of poor quality secondary to associated infection and relative ischemia such that clips and sutures may initially appear to close the defect-only to have it re-open at a later time (similar to what occurs during attempts at operative repair). Use of these closure devices should be limited to very small holes and those that are easily accessed endoscopically.

A novel treatment for gastrointestinal leaks is the use of endoscopic vacuum therapy (EVT) which involves the placement of a wound vac sponge attached to a naso- gastric tube into or adjacent to the leak. Continuous suction from a wound-vac device is then applied through the NG tube. The sponge induces the formation of granulation tissue while the suction removes fluid, pus, and any associated necrotic debris in order to induce leak healing. This treatment requires placement of a new

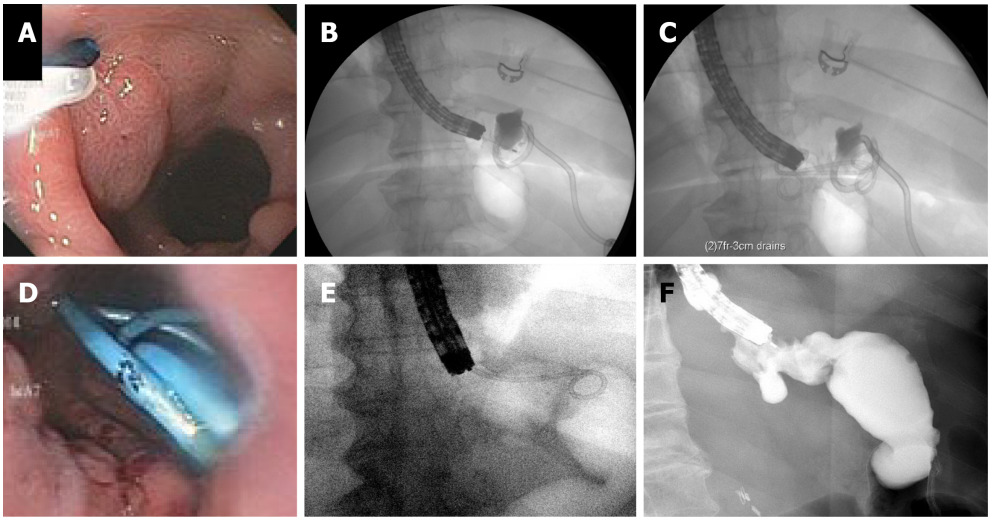


Figure 5 Sleeve gastrectomy leak treated with internal drainage. A: Endoscopic appearance of leak site; B: Contrast injection to confirm leak site; C: Placement of transgastric double pigtail stents; D: Endoscopic appearance of stents; E: Repeat endoscopy for stent removal; F: Contrast injection after stent removal confirming no residual leak.

sponge every 3-7 d, resulting in multiple endoscopic procedures. While the sponge is in place the patient cannot have any PO intake, therefore typically a PEJ tube should be placed for enteral nutrition. Several small retrospective case series have demonstrated the efficacy of this treatment for sleeve gastrectomy leaks. One single center series evaluated 9 patients who all had resolution of their leak after a mean of 50 d of treatment[63]. Another series demonstrated leak resolution in 7 of 8 patients, and a third series of 3 patients demonstrated resolution in all 3 cases with a mean treatment time of 72 d[64]. While EVT appears to be an effective treatment it is significantly uncomfortable for the patient and typically requires many endoscopies for sponge exchanges resulting in this primarily being a therapy for refractory or very large leaks not amenable to other therapies.

Sleeve stenosis

Stenosis of the gastric sleeve occurs in up to 4% of patients and typically occurs at the level of the incisura[65]. Patients typically present with symptoms of nausea and vomiting when advancing their diet beyond liquids. Other patients can present with symptoms of refractory gastroesophageal reflux. The diagnosis can often be made through upper GI series which will typically demonstrate an area of significant narrowing at the level of the incisura within the gastric sleeve. The diagnosis can also be made during upper endoscopy, but the endoscopist must be familiar with the condition - the narrowing is not typically a true mucosal stricture that prevents scope passage but rather an area of relative narrowing often with significant angulation requiring scope manipulation to get through. Contributing factors to the development of the stenosis include forming the sleeve around a small bougie, over-sewing the staple line, and inadvertent rotation of the sleeve during stapling can result in a twisted and narrowed sleeve[66].

Endoscopic treatment of sleeve stenosis involves the use of balloon dilation of the stricture. Dilation can be started at 20 mm with a radial expanding balloon. However, if dilation with the 20 mm balloon does not have much apparent effect on the stenosis then dilation can immediately proceed to pneumatic dilation with a 30 mm achalasia balloon under fluoroscopic guidance (Figure 6). Inflation to the 20 PSI maximum inflation pressure may not be possible during the first session. The patient can then return every 2 wk for repeat dilations with gradually increased inflation pressures and balloon sizes until the 40 mm balloon is used. The addition of dilating the pylorus up to 20 mm can also be helpful in improving symptoms. Dilations can be stopped once the patient is symptomatically improved. Efficacy of this dilation regimen was shown to be 76% effective in a large meta-analysis[67]. Complications of this treatment include bleeding or perforation and occur in about 6 % of patients[68]. Patients who fail endoscopic dilation will require either surgical seromyotomy or conversion to RYGB.

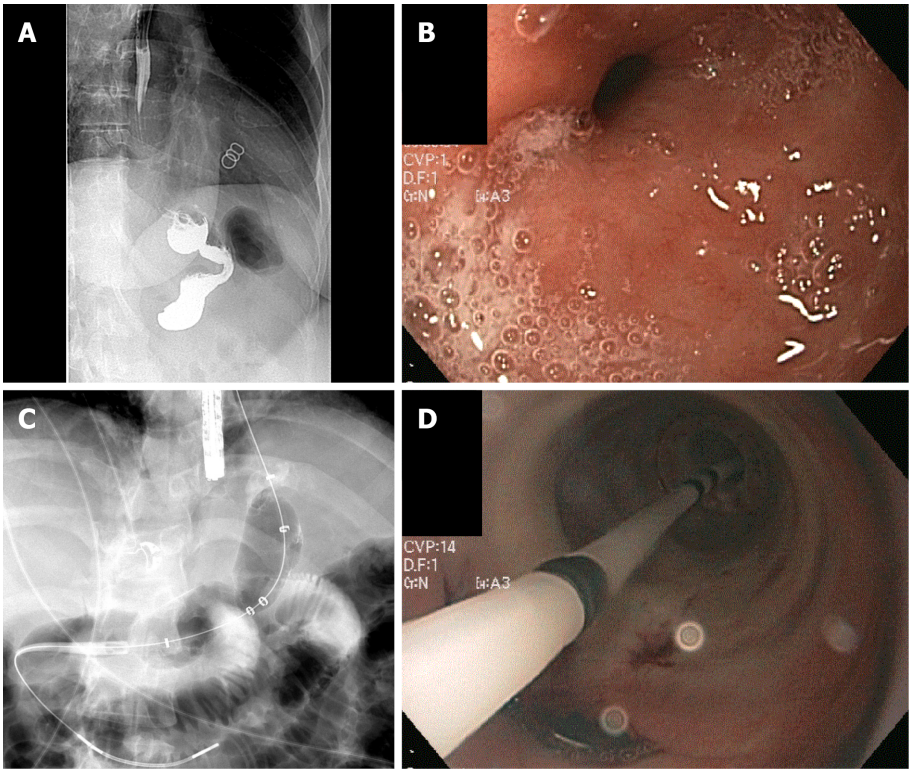


Figure 6 Sleeve stenosis treated with balloon dilation. A: Upper gastrointestinal series demonstrating stenosis at the level of the incisura; B: Endoscopic appearance of the stenosis; C: Pneumatic balloon dilation of stricture; D: Endoscopic appearance of balloon dilation.

LAGB

At the beginning of the century the LAGB was the most popular bariatric surgery in the United States. However, the use of this device has dramatically reduced secondary to poor efficacy and high numbers of complications, and it now represents less than 10% of bariatric surgeries performed nationwide. Many patients still have these bands in place and will continue to have adverse events needing endoscopic therapy. LAGB surgery involves the placement of a silicone band around the cardia of the stomach which creates a small proximal pouch to create a feeling of satiety after eating small amounts of food. The band is attached to a port that is placed in the subcutaneous tissue of the abdomen *via* a thin piece of tubing. The port can be used to inflate or deflate the band with saline to adjust the restrictive effect of the device. Common adverse events that occur with the LAGB include the development of GERD, esophageal dilation, port infection, band slippage and band erosion.

Band erosion

Placement of LAGB can be complicated by erosion of part of or the entire band into the gastric lumen. This can occur in the early post-operative period or many years later. Erosion of the band is thought to occur secondary to an inflammatory reaction between the band and the gastric wall. Precipitating factors include perforations at the time of surgery, bands placed too tightly around the stomach, bands that are sutured to the stomach and port infections[69]. Symptoms vary from patients being completely asymptomatic to the development of GERD, nausea and vomiting, or signs of infection [70]. Bands that have a significant portion visibly eroded into the gastric lumen can be removed endoscopically. If the band buckle is visible the likelihood of successful removal is greater. If the band appears to be insufficiently migrated, then placement of a covered esophageal stent for 2 wk can be considered to promote additional migration of the band[71]. Prior to endoscopic removal the subcutaneous port must be removed surgically- if necessary, this can be done simultaneously in the endoscopy suite.

The technique for endoscopic removal of the LAGB involves the use of the emergency mechanical lithotripter typically used for ERCP[72] (Figure 7). Initially the scope is passed through the center of the band, and a wire is placed into the distal stomach. The scope is then withdrawn, leaving the wire in place, and then re-

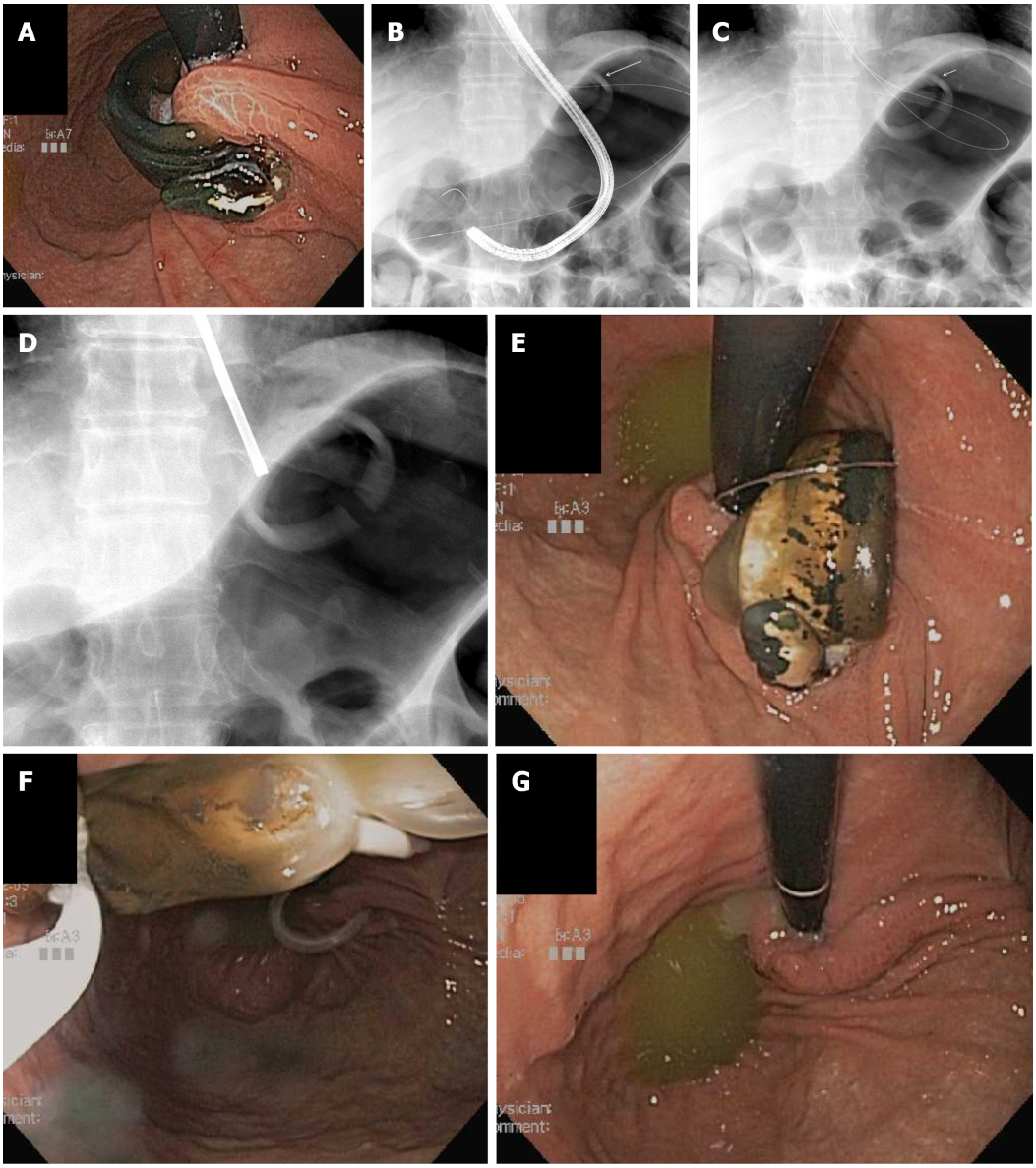


Figure 7 Eroded lap band removed endoscopically. A: Eroded lap band; B: Endoscope passage through the band with distal deployment of wire; C: Wire looped around band; D: Fluoroscopic view of cut lapband; E: Endoscopic view of cut lap band; F: Cut band grasped by snare; G: Endoscopic view after band removal.

introduced alongside the wire. The scope must be driven into the distal stomach without going through the center of the band, and the end of the wire is then grasped with a snare and pulled out through the mouth forming a loop around the band. The sheath of the mechanical lithotripter is then advanced over 2 ends of the wire up to the band. We use a combination of endoscopic and fluoroscopic imaging to ensure that only the band is trapped in the wire to avoid significant tissue trauma. The lithotripter is then cranked until it pulls the wire through the band to transect it. The lithotripter is then removed, and the scope is re-advanced into the stomach. If visible, the cut side with the buckle is grasped and used to pull the band out through the mouth[72]. The body forms a capsule around the band which prevents a perforation from occurring after removal. Adhesions can occur between the band and adjacent structures which prevent successful endoscopic removal. Bands that cannot be removed successfully endoscopically do need surgical removal.

CONCLUSION

The prevalence of obesity continues to rise around the globe, resulting in 1 of the most significant health problems affecting the world. Bariatric surgery rates will continue to climb in order to combat this disease. Despite improved surgical techniques, complic-

ations after these major surgeries are not uncommon. Post bariatric surgery patients are complex, and these complications are best managed with a multidisciplinary team with experience in this field. The endoscopic armamentarium continues to expand and helps serve as a minimally invasive treatment option for many patients with post-bariatric surgery complications.

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MINIREVIEWS

Update on the applications and limitations of alpha-fetoprotein for hepatocellular carcinoma

Hira Hanif, Mukarram Jamat Ali, Ammu T Susheela, Iman Waheed Khan, Maria Alejandra Luna-Cuadros, Muzammil Muhammad Khan, Daryl Tan-Yeung Lau

ORCID number: Hira Hanif 0000-0002-1888-0056; Mukarram Jamat Ali 0000-0003-3313-9933; Ammu T Susheela 0000-0002-4032-3133; Iman Waheed Khan 0000-0002-2772-2906; Maria Alejandra Luna-Cuadros 0000-0001-9259-2565; Muzammil Muhammad Khan 0000-0003-1324-3637; Daryl Tan-Yeung Lau 0000-0003-4139-1987.

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Hira Hanif, Mukarram Jamat Ali, Iman Waheed Khan, Maria Alejandra Luna-Cuadros, Muzammil Muhammad Khan, Daryl Tan-Yeung Lau, Liver Center, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Ammu T Susheela, Internal Medicine, Loyola MacNeal Hospital, Berwyn, PA 60402, United States

Corresponding author: Daryl Tan-Yeung Lau, MD, MSc, Associate Professor, Liver Center, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 110 Francis St. Liver Research Center, Suite 4A, Boston, MA 02215, United States. dlau@bidmc.harvard.edu

Abstract

Alpha-fetoprotein (AFP) is an oncofetal glycoprotein that has been used as a tumor marker for hepatocellular carcinoma (HCC) in combination with ultrasound and other imaging modalities. Its utility is limited because of both low sensitivity and specificity, and discrepancies among the different methods of measurements. Moreover, its accuracy varies according to patient characteristics and the AFP cut-off values used. Combination of AFP with novel biomarkers such as AFP-L3, Golgi specific membrane protein (GP73) and des-gamma-carboxyprothrombin significantly improved its accuracy in detecting HCC. Increased AFP level could also signify severity of hepatic destruction and subsequent regeneration and is commonly observed in patients with acute and chronic liver conditions and cirrhosis. Hereditary and other non-hepatic disorders can also cause AFP elevation.

Key Words: Alpha-fetoprotein; Hepatocellular carcinoma; Alpha-fetoprotein-L3; Cirrhosis; Tumor markers; Hereditary persistence of alpha-fetoprotein

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Core Tip: Alpha-fetoprotein has been used commonly as a biomarker for hepatocellular carcinoma (HCC) surveillance. Its sensitivity and specificity can be affected by the assay methods, patient characteristics and severity of the underlying liver conditions.

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Combination with other novel markers has shown promising results. Algorithms integrating these serum markers with noninvasive diagnostic imaging modalities are essential for the accurate and timely diagnosis of HCC.

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INTRODUCTION

During fetal and neonatal life, alpha-fetoprotein (AFP) is produced by yolk sac, fetal liver and gastrointestinal tract. Only a trace amount of AFP can be measured in adults due to its rapid decline in adults[1-4]. The use of AFP as a tumor marker for hepatocellular carcinoma (HCC) was first proposed in 1960s. The utility of AFP as a surveillance and diagnostic test has been criticized due to both low sensitivity and specificity. AFP, however, continues to play a significant role for HCC surveillance in combination with ultrasound (US) and other imaging modalities[5-8]. This review focused on both the clinical roles and limitations of AFP. In addition, the patterns of AFP elevation in various non-HCC etiologies were discussed.

BIOLOGY OF AFP

AFP is an oncofetal glycoprotein consisting of a single polypeptide chain of approximately 600 amino acids and nearly 4% carbohydrate. With molecular weight of 67500 dalton, it is a negatively charged protein with an isoelectric point of pH 4.57 and displays several charge isomers by extended agarose gel electrophoresis[1,2]. During the first trimester, AFP is mainly produced by yolk sac. After the atresia of yolk sac by 11-12 wk, fetal liver becomes the predominant source of AFP. A trace amount is also produced by the gastrointestinal tract of the fetus[1]. AFP in fetal serum can be detected as early as 29 d after conception and reaches its peak value of 3.0×10^6 ng/mL by 14th week of gestation. It declines to 2.0×10^5–3.0×10^5 ng/mL by week 32 and further decreases to 20-120 ng/mL at term[3]. By six months of life, serum AFP level is approximately 30 ng/mL. Thereafter, it is detectable between 3 and 15 ng/mL in adulthood[3,4].

AFP belongs to the family of serum albumin and the genes of which are present on the chromosome 4. It shares structure and physiochemical properties with its family members namely albumin, vitamin D-binding protein and afamin[9]. As both albumin and AFP show significant amino acid homologies, AFP is suggested to be an embryonic analogue of albumin[10]. Furthermore, because of its similarity to albumin, it has been postulated that AFP could be a carrier protein. In addition, its role as an immune-regulator and as a carrier protein for bilirubin has also been suggested[11].

AFP, being a principal serum binding protein in the fetus, plays a vital role in carrying various ligands such as fatty acids, hormones, minerals and bilirubin[12]. Though it performs many physiological functions during fetal development, elevated levels in adults are frequently observed in liver carcinogenesis and various disease processes[13-15]. Many biologic studies support the pro-oncogenic as well as anti-apoptotic effects of AFP. AFP can stimulate cell proliferation, cell motility and invasive properties of HCC cell lines and there is evidence that apoptosis of cancer cells could be achieved by blocking AFP[16,17]. On the other hand, a number of cancerous and non- cancerous causes involving both the liver and other organs can also lead to AFP elevation. AFP, therefore, is a non-specific marker of HCC[7] (Table 1).

HOW IS AFP MEASURED?

AFP was originally measured by immunoelectophoresis, but this method was not very

Table 1 Causes of alpha-fetoprotein elevation[13-15]

Hepatic	Non-hepatic
Neoplastic	**Neoplastic**
Hepatocellular carcinoma	Germ cell tumors (testicular and ovarian malignancies)
Intrahepatic cholangiocarcinoma	Gastric cancer
Non-neoplastic	**Non-neoplastic**
Liver cirrhosis	Normal pregnancy/infancy
Fulminant acute hepatitis	Colitis
Acute and chronic viral hepatitis	Fetal disorders (Gastroschisis, Neural tube defect)
Chronic liver diseases	Ataxia telangiectasia
Biliary obstruction (Intrahepatic and extrahepatic causes)	Hereditary tyrosinemia type 1
Drug induced hepatitis	Hereditary AFP persistence
Alcohol liver disease	Beckwith-Wiedemann syndrome
Non-alcoholic liver disease	Systemic lupus erythematosus
Neonatal hepatitis	Hirschsprung's disease
Massive hepatic necrosis	
Wilson disease	
Hemochromatosis	

AFP: Alpha-fetoprotein.

sensitive. In the 1970s and 1980s, new techniques such as radioimmunoassay and enzyme immunoassays were used. Subsequently, a quantitative automated chemiluminescent enzyme immunoassay was developed, which replaced and refined the previous clinical assays[18,19]. In this method, the serum sample is placed on a magnetic plate which is already bound by an anti-AFP antibody. A second chemiluminescent detection antibody, added to the same magnetic plate, then binds to all the AFP that is present in excess. All the unbound detection antibody is washed off and an organic substrate called as developer is added which emits light and become luminescent (Figure 1A). A chemiluminometer is used to detect the antibody, and quantification of the results is done against the known AFP standards. However, measurement interference may occur. In a single step method of AFP detection, sometimes interfering antibodies would bind to both the capture and detect antibodies leading to a false positive result (Figure 1B). Conversely, the interfering antibodies may blind to the reagents and inhibiting the proper interaction of AFP with the specific anti-AFP antibodies[20] (Figure 1C).

AFP as a diagnostic marker for HCC

The association between AFP and HCC is well recognized; however, the sensitivity and specificity of the AFP assay varies according to patient characteristics, design of the study, and the AFP cut-off values used[21,22] (Table 2). A systematic review of studies using AFP at a threshold value of 20 ng/mL in patients with cirrhosis, the sensitivity and specificity of detecting HCC were 41%-65% and 80%-94%, respectively [22]. Lowering the cut-off can increase the sensitivity of AFP but at the cost of higher false positives. By increasing the AFP value from 20 to 50 ng/mL, the specificity increased to 96% with a positive predictive value of 75% but sensitivity was reduced to 47%[23]. AFP values also vary according to the tumor size. The correlation of AFP to the size of the tumor was evaluated by Saffroy *et al*[24]. The bigger tumors generally had higher AFP values. The sensitivity of AFP, therefore, decreased from 52% for HCC > 3 cm to 25% for those < 3 cm in diameter[24].

For AFP-producing HCC, AFP plays an important role in initiating monitoring and terminating HCC therapy. A complete response of the treatment can be expected if preceding levels of AFP decrease to normal on follow ups. AFP, however, cannot be applied to monitor treatment response if its levels were not elevated prior to treatment [25-27]. Approximately one-third of the patients with HCC have no AFP elevation[28].

Table 2 Sensitivity and specificity of alpha-fetoprotein at different cut-off values[22-24]

AFP cut-off value	Sensitivity	Specificity
20	Approximately 60%	90%
50	47%	96%
100	31.2%	98.8%
400	17%	99.4%

AFP: Alpha-fetoprotein.

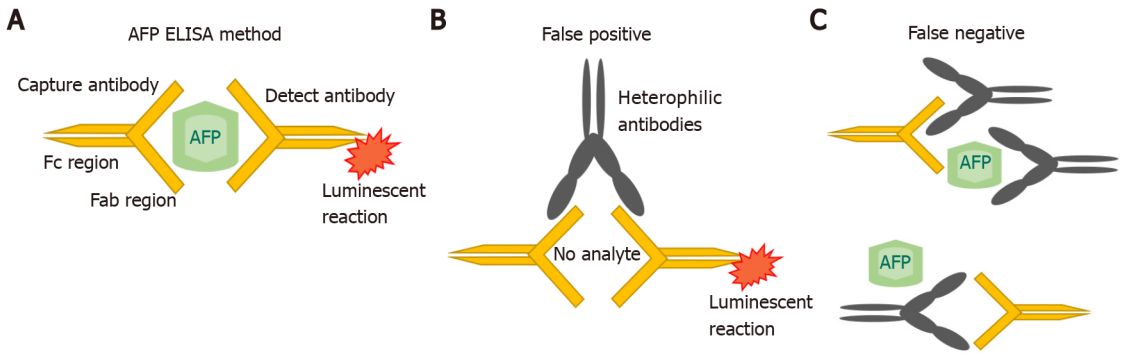


Figure 1 ELISA measures. A: ELISA measures alpha-fetoprotein (AFP) by detecting reactivity with two anti-AFP antibodies; B: Interfering antibodies that bind directly to the capture and detect reagents without the target AFP (no analytes). This binding would emit light and become luminescent causing false positive results and high values; C: Interfering antibodies can inhibit reactivity of the ELISA by binding to the reagents and prevent the detection of AFP by the anti-AFP antibodies; that leads to a false negative result. AFP: Alpha-fetoprotein.

Nomura *et al*[29] characterized those HCCs with low or no elevation of AFP. These patients generally have favorable prognosis with low probability of HCC recurrence and improved survival as compared to those with elevated AFP levels[29,30]. In a large retrospective study including 1800 patients with HCC, 42% had AFP < 20 ng/mL whereas 16% had AFP levels between 20 and 100 ng/mL. Thus, a total of 58% had AFP < 100 ng/mL. The authors observed that 67% of patients with HCC smaller than 5 cm had AFP ≤ 100 ng/mL *vs* only 49% of the patients with HCC larger than 5 cm had AFP ≤ 100 ng/mL. In addition, HCCs without AFP elevation (≤ 100 ng/mL) were less likely to develop portal vein thrombosis[31].

AFP-L3 and other HCC biomarkers

Based on the binding capacity of AFP to lens culinaris agglutinin (LCA), three separated bands of AFP glycoforms can be identified with the Western blotting. These are AFP-L1 (non-binding), AFP-L2 (intermediate binding) and AFP-L3 (LCA-reactive). AFP-L1 generally correlates with hepatic inflammation in chronic hepatitis while AFP-L3 is more specific for HCC. AFP-L2 is derived from yolk sac and is detectable in the maternal serum during pregnancy[32] (Table 3).

AFP-L3 is calculated as a fraction of AFP-L3 to total AFP. The cut-off values most commonly used for a positive AFP-L3 test for patients with HCC is 10% though levels above 15% are more specific[33]. It is secreted in the initial tumor stages and thus can be used as an early HCC diagnostic marker[32,34]. AFP-L3 level can also distinguish the histological differentiation of tumors[25]. In patients with total AFP level ≤ 200 ng/mL, AFP-L3 specificity may reach 100% for HCC if it increases more than 35% of the total AFP[35]. In a large multicenter prospective study of hepatitis C virus related HCC, the specificity of AFP-L3 was observed to be 92% but its sensitivity was fairly low at 37%, irrespective of the tumor stage[36]. On the contrary, a number of recent studies reported a much higher sensitivity of AFP-L3 for diagnosing HCC[37-39]. Ibrahim *et al*[39] evaluated the diagnostic efficacy of AFP-L3 among 20 healthy individuals, 40 patients of chronic active hepatitis and 40 HCC patients with underlying chronic hepatitis B (CHB) or C. Among the HCC patients, 30 (75%) had tumor size > 5 cm. At a cut-off point of > 12.3 ng/mL, AFP-L3 was 100% accurate in diagnosing the HCC[39]. In a study by Ibrahim *et al*[39], 20 healthy individuals, 20 chronic liver disease patients and 40 patients HCV-induced HCC were included in the

Table 3 Overview of the diagnostic parameters for hepatocellular carcinoma

Diagnostic parameter (cut off)	Remark	AUROC
AFP (> 20 ng/mL)	Sensitivity: 40%–65%	0.54–0.80
	Specificity: 76%–96%	
AFP-L3 (> 15%)	Sensitivity: 45%–90%	0.74–0.84
	Specificity: 95%	
AFP-L3 + AFP-P4 + P5 (> 15% for both biomarkers)	Sensitivity: 55.3%–61%	0.76
	Specificity: 82.3%–93.9%	
AFP, AFP-L3 and DCP (3-25 ng/mL, 1%-10%, 0.48 ng/mL or 40 mAu/mL)	Sensitivity: 81%–93%	0.88–0.93
	Specificity: 69%-87%	
AFP and GP73	Sensitivity: 75%-91%	0.91–0.95
	Specificity: 81%-97%	

AFP: Alpha-fetoprotein; DCP: Des-gamma-carboxyprothrombin.

analysis. A large proportion (65%) of the HCC patients had advanced stage of HCC. By using a cut-off value of 23 ng/mL, AFP-L3 had a sensitivity of 97.5% and specificity of 100% in predicting HCC at[37]. Cerban *et al*[38] investigated the diagnostic performance of AFP, AFP-L3, PIVKA-II and GPC-3 for HCC in a cohort including 52 cirrhotic and 101 HCC patients. AFP-L3 at a cut-off point of > 13.5 ng/mL was found to have a higher sensitivity of 84.7%, compared to other biomarkers[38]. The sensitivity of AFP-L3 for HCC detection can be affected by its cut-off values, as well as the tumor size and stage[40]. Data suggested that AFP-L3 could potentially be a valuable prognostic tool. The persistent elevation of AFP-L3 after HCC therapy was found to be associated with shorter survival[25].

Recently, a highly-sensitive AFP-L3 or hs-AFP-L3 was developed. Toyoda el al. determined the performance of hs-AFP-L3 among patients with 44 patients with chronic hepatitis or cirrhosis, and 54 patients with HCC. The sensitivity and specificity of hs-AFP-L3 were 84.9% and 88.6%, respectively[41].

Since both total AFP and AFP-L3 have limitations for the detection of HCC, studies have been conducted to combine AFP and AFP-L3 with other novel immunoassays to improve their performance. Des-gamma-carboxyprothrombin (DCP), also known as PIVKA-II (Protein Induced by vitamin K absence or antagonist-II), is a promising HCC biomarker. PIVKA-II is an abnormal product of liver carboxylation that reflects oncogenesis and HCC progression and is undetectable in healthy patients[25,42].

A systematic review noted that AFP, AFP-L3 combined with DCP enhanced their individual HCC predictive values with pooled sensitivity and specificity of 88% and 79%, respectively[43,44]. Since 2002, Japan has included these 3 biomarkers in their HCC surveillance programs and they noted improved rates of early HCC identification and prognosis[43]. When applying the GALAD score (age, sex, AFP, AFP-L3 and DCP), the HCC diagnostic accuracy was even higher [area under curve (AUC) 0.976][45,46]. A recent meta-analysis reported that AFP combined with GP73 (a Golgi specific membrane protein found in epithelial cells of bile ducts) has favorable sensitivity (84%) and specificity (92%) in diagnosing HCC with an AUROC value of 0.93[47].

Lately, a new HCC diagnostic marker, the long noncoding RNAs (lncRNAs), was developed[48]. LncRNAs are transcribed by RNA polymerase II and play a role in the regulation of transcription and translation of proteins. Small extracellular vesicles (EV) transfer proteins, DNA, and RNA between tumor and nontumor cells. The dysregulated lncRNAs including EV-derived lncRNAs contribute to HCC progression and metastasis. EV-derived lncRNAs are, therefore, potential novel serum biomarkers. Kim *et al*[48] evaluated LINC00853, an EV-derived lncRNA in 90 patients with HCC and 92 without HCC in a cohort study. The AUROC of LINC00853 was 0.935 for identifying all-stage HCC and 0.969 for detecting early-stage HCC. These results were significantly better compared to AFP[48]. In another study by Yu *et al*[49] reported that the combination of AFP with 2-lncRNA had a higher discriminative power than that of AFP alone in the diagnosis of HCC[49].

AFP ELEVATION IN LIVER DISEASES AND CIRRHOSIS

Rise in AFP has been recorded in different chronic liver diseases without HCC and other malignancies, hence, the results of AFP levels should be interpreted with caution [50]. AFP is known to have a role in liver regeneration, inflammation and liver fibrosis. Less pronounced AFP elevation > 10 ng/mL has been reported in 15%-58% of chronic hepatitis and 11%-47% of cirrhosis[51]. The usual cut off for differentiating benign *vs* malignant liver conditions was at 500 ng/mL but can be variable[52]. Studies have reported a progressive rise in serum AFP levels with increased histological severity from inflammation to cirrhosis to HCC[53].

ACUTE HEPATITIS

The degree of AFP elevation in acute hepatitis is related to the severity of hepatic destruction[50,54]. The levels range from 10 ng/mL-1000 ng/mL with occasional higher value from 3000 to 7190 ng/mL has been reported. Among children with acute hepatitis B, AFP was detected within 1 wk of the onset of clinical hepatitis and returned to normal by the time of recovery with loss of HBsAg[55]. The possible mechanisms of AFP elevation include acute phase reaction to the liver injury, hepatocyte regeneration, or viral control or mediated AFP synthesis[56]. AFP elevation after significant alanine aminotransferase (ALT) elevation is most likely due to liver regeneration and there is generally a latent period of 5-16 d[57]. AFP generally peaks at a time when liver destruction is subsiding and hepatic remodeling begins[57]. Hence, the highest level of AFP in acute hepatitis generally occurs during the recovery phase of illness and is an indicator of the liver regenerative process[58].

CHB

Elevated AFP levels are frequently observed during the course of CHB without HCC. The commonest cause of AFP elevation was the exacerbation of the underlying liver disease with or without changes in the status of hepatitis B virus (HBV) replication[41, 52]. High AFP levels associated with hepatitis flare were also found to be predictive of HBeAg to anti-HBe seroconversion[59,60]. AFP levels usually decrease within 12 mo on antiviral therapy[61]. Kim *et al*[61] reported that AFP normalization (< 20 ng/mL) was achieved in 89.5% of treated patients but remained abnormal in 40.6% of antiviral treatment naïve patients[61].

Persistent elevation of AFP > 100 ng/mL without a parallel increase in ALT level was reported to be a predictor of HCC with a sensitivity of 98.7% and specificity of 66.7%[59]. Yuan *et al*[62] conducted a retrospective study of 302 treatment naive CHB patients with AFP positive status. After antiviral therapy, they found that there was a 6.35-fold increased HCC risk among those with persistently elevated AFP compared to those with normalized AFP levels[62].

In a metanalysis, Peng *et al*[63] concluded that among HBV–related HCCs with low AFP levels, circulating miRNAs could be potential valuable biomarkers. For patients with AFP levels < 400 ng/mL, miR-125b and miR-205 demonstrated a sensitivity of 90% while combination of miR-15b and miR-130b had > 90% sensitivity and specificity. For AFP < 20 ng/mL, miR-26a, 27a, 7b and combination of miR-122 and miR-7b exhibited a sensitivity of 80% while combination of mir-29a, 29c, 133a, 143, 145, 192 and 505 produced a specificity of > 80%. Additionally, the combination of miR-15b and miR-130b showed high diagnostic accuracy with sensitivity and specificity exceeding 90%. For differentiating HCC patients with low AFP levels (< 20 ng/mL) from non-HCC patients, the overall sensitivity and specificity of miRNAs were 0.85 and 0.74, respectively with AUC of 0.88 (95%CI: 0.85–0.90). For patients with AFP < 400ng/mL, the overall sensitivity and specificity were 0.84 and 0.76 with AUC of 0.88 (95%CI: 0.84–0.90). Hence, miRNAs are attractive HCC markers to detect HBV-associated HCC with low AFP levels[63].

Dickkopf WNT signaling pathway inhibitor 1 (DKK-1) is a glycoprotein that is expressed in various malignancies. Shen *et al*[64] conducted a study on 424 HCC, 98 CHB, 96 cirrhosis and 213 healthy controls and measured their DKK-1 Levels. The diagnostic cut-off was established at 2.153 ng/mL. DKK-1 proved useful in the detection of AFP-negative HCC with 70.4% sensitivity and 90.0% specificity. These values were validated in another cohort. When combined with AFP, DKK-1 increased detection rate of early-stage HCC with 73.1% sensitivity and 90.0% specificity. Add-

itionally, elevated DKK-1 Levels can help differentiate HCC from other liver diseases such as CHB and cirrhosis with 69.1% sensitivity and 84.7% specificity[64,65].

Hepatitis B flare is defined as an abrupt elevation of serum ALT to 5 times the ULN or greater than 3-fold increase from baseline[66]. During hepatitis flare, peak AFP level was usually observed 1-2 wk after peak ALT rise. Significant AFP elevation to > 2500 ng/mL had been documented with hepatitis flare. The normalization of AFP might take up to 3-12 mo[66].

The presentation varies from asymptomatic to overt hepatitis with decompensation and liver failure. AFP elevation was noted in 25%-30% of the patients with hepatitis flares. A study reported the annual incidence of hepatitis flares to be 27% in HBeAg-positive patients and 10% in HBeAg-negative patients with a mean follow-up period of 2 years. Chang *et al*[67] reported that patients with AFP > 100 ng/mL during flare cleared HBeAg at a rate of 31% in 3 mo, 62% in 12 mo and 72% in 18 mo. In contrast, the corresponding HBeAg clearance rates for those with AFP < 100 ng/mL were only 4%, 15% and 19% in 3, 12 and 18 mo respectively[67]. Patients with bridging hepatic necrosis (BHN) had increased activation of the AFP-producing oval cell. BHN was noted in 80% of the cases with AFP levels > 100 mg/dL[53]. BHN, AFP > 100 ng/mL and decreasing HBV DNA titers were identified as good prognostic indicators of an effective immune control with HBV DNA suppression and/or HBeAg clearance.

Severe and repeated hepatitis flares could also lead to development of cirrhosis or hepatic decompensation. Patients with severe hepatitis flare with BHN or AFP > 100 ng/mL but fail to suppress HBV DNA should be treated promptly with antiviral therapy.

CHRONIC HEPATITIS C

AFP elevation is more greatly associated with HCV-associated HCC than HBV-associated HCC[64]. The incidence of elevated AFP in chronic hepatitis C (CHC) patients range from 10%-43%[61]. Elevated AFP > 20 ng/dL in CHC is associated with female gender, black race, increased age, genotype 1b, low albumin level, elevated aspartate aminotransferase (AST), elevated AST/ALT ratio, low platelet, prolonged PT, and increased ferritin levels[68,69]. Yang *et al*[70] evaluated 279 CHC patients and found no correlation between AFP and the levels of HCV RNA[70]. In the HALT C (Hepatitis C Antiviral Long-term Treatment) study, Di Bisceglie *et al*[71] observed AFP > 20 ng/mL in 16.6% of patients without HCC; it was associated with cirrhosis, female and Black patients[71]. Black patients with CHC tend to have higher AFP elevation compared to other racial groups[71]. Generally, HCV-related HCC does not have significant AFP elevation. Thus, AFP has especially low sensitivity to identify HCC in Black populations[71].

Fouad *et al*[72] reported that sustained virological response with direct-acting antiviral agents (DAA) therapy was associated with a significant reduction in serum AFP and might be used as a predictor of treatment response[72]. In another study, 60% of the DAA treated patients normalized AFP compared to only 23% without therapy [61].

Therapeutic phlebotomy has also been reported to reduce AFP levels in CHC[44]. It has been postulated that iron mediated oxidative stress is associated with hepatic injury; iron depletion decreases the oxidative stress and indirectly lowers the AFP level[73].

NONALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in the Western world with about 10%-30% progress to cirrhosis[74]. It is estimated that 6 million people in the United States have NASH with 1%-2% incidence of HCC annually. Published reports estimated that over 25% of NASH-related HCC occurred in patients without cirrhosis. With the high prevalence of NAFLD, risk stratification is essential to implement HCC surveillance programs[75]. Currently, no surveillance of NALFD patients for HCC is recommended by AASLD[76]. However, it has been suggested that NAFLD patients with cirrhosis or possible fibrosis or diabetes mellitus should have 6 monthly surveillances *via* ultrasound (US) and tumor markers (AFP, AFP-L3 and DCP). Abdominal fat can be a hindrance, hence, patients with obesity can be alternatively surveilled using CT-Scan or magnetic resonance imaging[77].

An association between AFP elevation and NAFLD was first established by Babalı *et al*[78] in 2009. This study demonstrated a significant increase in AFP levels in patients suffering from NAFLD (4.09 ± 1.68) in comparison to the control group (2.95 ± 0.41) with P value < 0.05.Additionally, three subgroups of NAFLD were created based on the liver ultrasonography findings with presence of hepatorenal contrast or bright liver corresponding to grade 1, both hepatorenal contrast and bright liver signifying grade 2, and both pre-mentioned findings with bright liver being severe representing grade 3. The results showed a significant increase in AFP in grade 3 NAFLD (5.43 ± 1.51) in comparison to grade 2 (3.97 ± 1.45) and grade 1 (2.92 ± 1.06) with a P value of 0.001. Triglyceride, cholesterol, low density lipoprotein, high-density lipoprotein, glucose and ALT levels were not significantly different in these groups[78].

Best *et al*[79] published a case control study on German centers in 2020 describing the relationship between HCC and NASH using AFP, AFP-L3, DCP and the GALAD score for assessment. 125 NASH patients with HCC and 231 NASH control patients without HCC were taken. The GALAD score recognized patients with HCC at any stage with an AUC of 0.96 which was superior to AUC of AFP (AUC 0.88), AFP-L3 (AUC 0.86) and DCP (AUC 0.87) levels with P < 0.001 at a cut off about -0.63. The sensitivity was 84.8% and specificity was 95.2% at this cut off. They also conducted Japanese cohort study on 392 patients with NAFLD out of which 28 patients developed HCC. The mean GALAD score in these 28 patients predicted occurrence of HCC 1.5 years before its diagnosis. GALAD score was considerably higher in these patients compared to NASH patients who did not develop HCC[79].

EARLY *VS* ADVANCED LIVER CIRRHOSIS WITH DECOMPENSATION

Arrieta *et al*[80] reported that AFP ≥ 200 and 400 ng/mL had a sensitivity of 36.3% and 20.2% in determining HCC with 100% specificity. Improved method of analysis using progressive elevation of AFP ≥ 7 ng/mL/mo among cirrhotic patients increased the sensitivity to 71.4% with 100% specificity. This method was successful even when AFP values were below 200 ng/mL[80].

AFP can be elevated in patients with CHC or cirrhosis without any evidence of HCC [81]. A persistent AFP value of 17.8 ng/mL was noted to have a 98.6% specificity, 35% sensitivity and a positive predictive value of 97.7% in identifying patients with cirrhosis[82]. Sudden fluctuations in AFP levels in patients with cirrhosis can be indicative of hepatitis flare, deterioration of liver disease and development of HCC [83]. AFP has been applied routinely for HCC surveillance in cirrhotic patients. Harada *et al*[83] showed that 40% of cirrhotic patients had AFP levels > 20 mg/dL[83]. Manuc *et al*[84] reported in a study of 2068 CHC patients with cirrhosis, 30.1% without HCC had AFP > 15 ng/mL due to advanced age, severe liver injury with raised AST and ALT levels and low platelets[84].

In patients with cirrhosis, both the sensitivity and specificity of AFP for detection of early-stage HCC diagnosis can be enhanced by combining it with US. In a meta-analysis, the pooled sensitivity of AFP and US for any stage and early-stage HCC were 95% (83%-100%) and 60% (45%–74%) respectively, compared to only 72% (56%–86%) and 40% (22%–58%) for US only[85]. Surveillance with AFP and ultrasonography in cirrhotic patients having an annual risk of HCC > 0.4% is cost-effective[86].

There are many risk scores that have been developed to predict HCC development among patients with chronic hepatitis and cirrhosis. However, many of them have not been standardized or carefully validated in different patient populations. Ideally, HCC prediction models should be developed for treated and untreated patients taking into account the patient ethnicity, significant comorbid conditions and ideally the molecular biomarkers[87].

PERSISTENT AFP ELEVATION IN THE ABSENCE OF LIVER DISEASE

AFP is an important screening test for HCC and is also used in the diagnostic evaluation of other hepatic and non-hepatic conditions. Persistent AFP elevation has also been reported in patients without malignant or nonmalignant conditions. Hereditary persistence of AFP (HPAFP), a benign autosomal dominant disorder with no apparent disease or abnormality, should be considered as one of the differential diagnoses in patients with unclear etiology of persistent AFP elevation[88]. HPAFP is a rare condition with only 20 reported cases in the literature[89]. In contrast to malignant tumors with AFP usually > 500 ng/mL, the AFP concentration in HPAFP is mostly

below 200 ng/mL, but levels up to 1500 ng/mL have been reported in some cases[88,90,91]. The molecular mechanism of HPAFP can differ in unrelated families. Specific heterozygous point mutations are frequently found in the promoter region of the AFP gene related to hepatocyte nuclear factor 1 (HNF1) binding sites[92,93]. These mutations usually result in an increased affinity for HNF1 and subsequently lead to increased AFP promoter activity and AFP gene transcription[94]. Two-point mutations have been identified; the upstream substitution of cytosine (C) with adenosine (A) at amino acid position of 55 (-55 C > A) in the proximal HNF-1 binding site and upstream mutation caused by the substitution of Guanine (G) by Adenosine (A) at position 119 (-119 G > A) in the distal HNF-1 binding site[95,96]. The first case of HPAFP was reported in 1984 by Greenberg *et al*[91] in a Scottish family where a 38-year-old woman was noted to have persistently elevated AFP during post-partum[91]. The AFP concentration of her amniotic fluid was normal and HPAFP was later confirmed by evaluating serum AFP levels and molecular testing in family members. In another report of 20 HPAFP cases, 2 patients underwent unnecessary surgery and 3 had unnecessary chemotherapy due to their persistently elevated AFP[96]. Though rare, hereditary causes should be considered in patients with unexplained and persistent AFP elevation. In a recent study by Jeon *et al*[89] on 4 Korean patients with persistently elevated AFP levels from 12.1 to 186.1 ng/mL for > 1 year, 1 patient was found to have a hereditary cause by pedigree analysis even though the typical mutation of the AFP gene in the promoter region was absent. This case elucidated the heterogeneous nature of persistent AFP elevation and HPAFP is not always the result of mutation in the AFP transcription regulatory regions[89].

CONCLUSION

AFP has been used for HCC surveillance widely in clinical practice. It, however, has limited sensitivity and specificity for HCC detection, and a proportion of patients with advanced HCC do not have AFP secretion. Moreover, patients with chronic liver diseases, especially those with cirrhosis are commonly identified with persistent AFP elevation without radiological evidence of HCC. For all these reasons, AFP is not recommended to be used as a sole marker alone for HCC surveillance. The diagnostic potential of AFP for early diagnosis of HCC can be enhanced by combining it with other novel diagnostic markers such as AFP-L3, PIVKA-II, and GP73. Although many have already endorsed diagnostic value, large number of multicenter studies encompassing larger cohorts and long-term assessment are required to confirm clinical utility. Additionally, algorithms integrating these serum markers with noninvasive diagnostic imaging modalities are needed to be developed for the early and accurate diagnosis of HCC.

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ORIGINAL ARTICLE

Case Control Study

Obesity is associated with decreased risk of microscopic colitis in women

Robert S Sandler, Temitope O Keku, John T Woosley, Dale P Sandler, Joseph A Galanko, Anne F Peery

ORCID number: Robert S Sandler 0000-0002-1323-6245; Temitope O Keku 0000-0003-4019-3903; John T Woosley 0000-0001-5876-3068; Dale P Sandler 0000-0002-6776-0018; Joseph A Galanko 0000-0003-2304-4375; Anne F Peery 0000-0002-2005-0780.

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Robert S Sandler, Department of Medicine, University of North Carolina, Chapel Hill, NC 27514-7555, United States. rsandler@med.unc.edu

Temitope O Keku, Joseph A Galanko, Anne F Peery, Department of Medicine, University of North Carolina, Chapel Hill, NC 27514-7555, United States

John T Woosley, Department of Pathology, University of North Carolina, Chapel Hill, NC 27514, United States

Dale P Sandler, Department of Health and Human Services, National Institute of Environmental Health Sciences, Durham, NC 27709, United States

Corresponding author: Robert S Sandler, MD, MPH, Professor, Department of Medicine, University of North Carolina, No. 130 Mason Farm Road, Chapel Hill, NC 27599-7555, United States. rsandler@med.unc.edu

Abstract

BACKGROUND

Microscopic colitis is a leading cause of diarrhea in the older adults. There is limited information about risk factors. We hypothesized that obesity would be associated with microscopic colitis.

AIM

To examine the association between obesity and microscopic colitis in men and women undergoing colonoscopy.

METHODS

We conducted a case-control study at the University of North Carolina Hospitals. We identified and enrolled men and women referred for elective, outpatient colonoscopy for chronic diarrhea. We excluded patients with a past diagnosis of Crohn's disease or ulcerative colitis. A research pathologist reviewed biopsies on every patient and classified them as microscopic colitis cases or non-microscopic colitis controls. Patients provided information on body weight, height and exposure to medications *via* structured interviews or Internet based forms. The analysis included 110 patients with microscopic colitis (cases) and 252 non-microscopic colitis controls. Multivariable analyses were performed using logistic regression to estimate odds ratios and 95% confidence intervals.

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RESULTS

Cases were older and more likely than controls to be white race. Study subjects were well educated, but cases were better educated than controls. Cases with microscopic colitis had lower body mass index than controls and reported more weight loss after the onset of diarrhea. Compared to patients who were normal or under-weight, obese (BMI > 30 kg/m^2) patients were substantially less likely to have microscopic colitis after adjusting for age and education, adjusted OR (aOR) 0.35, 95% confidence interval (CI) 0.18-0.66). When stratified by sex, the association was limited to obese women, aOR 0.21, 95%CI: 0.10-0.45. Patients with microscopic colitis were more likely to report weight loss after the onset of diarrhea. After stratifying by weight loss, there remained a strong inverse association between obesity and microscopic colitis, aOR 0.33, 95%CI: 0.10 – 1.11 among the patients who did not lose weight. Ever use of birth control pills was associated with lower risk of microscopic colitis after adjusting for age, education and BMI, aOR 0.38, 95%CI: 0.17-0.84.

CONCLUSION

Compared to controls also seen for diarrhea, microscopic colitis cases were less likely to be obese. Mechanisms are unknown but could involve hormonal effects of obesity or the gut microbiome.

Key Words: Colitis; Microscopic/epidemiology; Humans; Diarrhea/epidemiology; Obesity

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Core Tip: We conducted a case control study among patients undergoing colonoscopy for diarrhea. The analysis included 110 patients with microscopic colitis and 252 controls. Obesity was associated with a substantially lower risk of microscopic colitis among women that was not explained by weight loss following the onset of diarrhea. Ever use of birth control pills was associated with lower risk of microscopic colitis after adjusting for age, education and BMI. The mechanism could involve hormonal effects of obesity or the gut microbiome.

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INTRODUCTION

Microscopic colitis is a chronic inflammatory disease of the colon characterized by a normal or near normal endoscopic appearance but abnormal pathology. It is increasingly recognized that endoscopically visible lesions can be recognized in nearly 40% of patients although they are non-specific[1]. Histologically, there is a thickened collagen band (collagenous colitis) or an increase in intraepithelial lymphocytes (lymphocytic colitis)[1]. Microscopic colitis was first described in 1976 by Lindstrom [2]. The term microscopic colitis was used by Read *et al*[3] in a 1980 publication describing a series of patients with chronic diarrhea of unknown origin. While initially considered uncommon, population-based studies have shown a rising incidence that may have started to plateau[4-6].

Microscopic colitis is now a frequent cause of chronic diarrhea, particularly in older adults. In some regions, the incidence of microscopic colitis exceeds Crohn's disease and ulcerative colitis[7]. Despite an increasing burden of disease, the etiology of microscopic colitis is not known. Prior studies implicated a range of medications including proton pump inhibitors (PPIs), nonsteroidal anti-inflammatory drugs (NSAIDs), statins, and beta blockers[8]. Cigarette smoking has also been implicated[9, 10]. The condition has been reported to be more common in patients with a number of auto-immune diseases[11].

There is currently limited information that obesity might be inversely associated with microscopic colitis[12,13]. We conducted a case-control study among a population of patients scheduled for colonoscopy due to chronic diarrhea in order to assess whether obesity and hormonal factors might be associated with microscopic colitis.

MATERIALS AND METHODS

Briefly, we identified male and female patients between April 1, 2015 and December 22, 2020 who were referred for outpatient colonoscopy for diarrhea. We excluded patients with a prior history of Crohn's disease and ulcerative colitis. Potential participants were mailed information about the study in advance of their procedure. On the day of their exam, eligibility was verified by a research assistant who obtained informed consent for participation. To be eligible for the study, patients had to report a Bristol Stool Form type 5, 6, or 7 (mushy, loose, watery) during the week prior to their colonoscopy regardless of the number of stools[14] . We recruited patients from each of the 3 endoscopy units at the University of North Carolina Chapel Hill. If the research assistant was not available for consent at the time of the procedure, the patient was later contacted to verify eligibility and obtain consent. We also queried the hospital pathology database every month and contacted patients with biopsy proven microscopic colitis who were not identified prior to their colonoscopy. This group included patients who were referred for colonoscopy for other reasons (generally screening) but reported diarrhea and had colon biopsies taken during the colonoscopy to assess for microscopic colitis. In sensitivity analyses, the patients identified retrospectively from pathology were excluded.

Patients with signs of gross inflammation on colonoscopy were excluded. Patients with subtle or isolated mucosal abnormalities were not excluded. Patients with non-lymphocytic colitis on biopsies were also excluded. A research pathologist (JTW) who was not aware of the clinical diagnosis reviewed the slides for all enrolled study subjects. Cases were patients with microscopic colitis on biopsy defined by increased number of intraepithelial lymphocytes. Additional features included increased lamina propria chronic inflammation, with minimal crypt distortion or active cryptitis. Collagenous colitis was defined by a thickened subepithelial collagen band. Slides were initially reviewed by a clinical pathologist. The slides were then re-read by the study pathologist. When there was a disagreement between the clinical pathologist and the research pathologist, the research pathologist re-read the slides. In addition, a 20% sample of slides were resubmitted to the research pathologist. After excluding indeterminate colitis, there was a 100% match between the initial and final reading by the research pathologist. Controls were patients with normal pathology. Patients with indeterminate microscopic colitis based on a sparse number of lymphocytes were excluded to avoid possible misclassification and because there were too few for separate analysis.

After the colonoscopy, all participants completed a 30 to 40-minute structured telephone interview or self-completed the same questionnaire using an internet-based form. The telephone interviewer verified eligibility, including the diarrhea criteria, for patients who were identified from pathology or who were missed in the endoscopy unit. Participants self-reported demographics, body weight, height, medical history including over-the-counter and prescription medications, reproductive history, bowel habits, and gastrointestinal symptoms. The reproductive history for women included questions about age at first menses, oral contraceptive use, gravidity, parity, and if postmenopausal, age at and type of menopause, and menopause hormone therapy use. All exogenous hormone questions included age at initiation, duration of use, and use in the last year, but not type of hormone or dose. BMI was calculated from self-reported weight in kilograms divided by height in meters squared (kg/m^2). BMI was categorized using Centers for Disease Control criteria: BMI < 25 kg/m^2 (under- or healthy weight), BMI 25.0 < 30 kg/m^2 (overweight), BMI > 30 kg/m^2 (obese).

Data analysis was conducted using Stata 17.0 (Stata Corp. College Station, TX). The analysis was reviewed by a biostatistician (JAG). Variables were examined one-by-one in bivariate analyses using chi square tests for categorical variables and Student t-tests for continuous variables. Multivariable logistic regression models were used to calculate adjusted odds ratios and 95% confidence intervals adjusted for age, educational status and BMI. Smoking was not included in models because smoking was not independently associated with microscopic after adjusting for education. We have previously examined the association between medications thought to be

associated with microscopic colitis (PPI, statins, SSRI, NSAIDs)[15]. There was no association in our study so those drugs were not included in models. Multivariable logistic models also included terms for reproductive factors including age at first live birth, number of live births and age of menarche. The model terms were informed by review of the relevant literature and directed acyclic graphs (DAGs)[16]. For analyses of menopause, the reference group was the most common age of natural menopause, age 45-54. Missing data were not imputed.

The study was approved by the University of North Carolina Office of Human Research Ethics. All patients gave informed consent.

RESULTS

Patients were enrolled in the study between April 1, 2015 and December 22, 2020. Among the 1008 patients who were referred for colonoscopy for diarrhea, 176 cancelled their colonoscopy, 161 were ineligible, 99 were missed in the endoscopy unit and not subsequently recruited, and 196 refused. After excluding indeterminate colitis (n = 14), there were 362 who serve as the basis of this report. There were 110 microscopic colitis cases (including 34 identified from pathology reports) and 252 controls. Interviews were completed by phone by 84% of subjects and Internet for the remainder.

Table 1 shows characteristics of the study population. The cases were older than controls: case mean age 63.2 (standard deviation (SD) 12.7, interquartile range 53-73) *vs* control mean age 54.5 (SD 11.8, interquartile range 45-64). Cases were more likely than controls to be white (96.2% *vs* 85.7%). As a group, the study subjects were very well educated, but cases were more likely to have a college degree or have attended graduate school, 66.0% *vs* 44.3%. Cases were less likely to be current smokers, 11.3% *vs* 21.0%. There was a strong correlation between smoking and education, with better educated patients substantially less likely to smoke (not shown). There was no difference between cases and controls for marital status, race, or smoking after adjustment.

Overall, mean BMI was lower in cases (BMI 25.7 kg/m^2 (SD 6.4)) than controls (BMI 29.5 kg/m^2 (SD 7.2)). As shown in Table 2, the risk for microscopic colitis was lower for BMI 25-30 kg/m^2 (OR 0.71, 95%CI: 0.40-1.25) and BMI > 30 kg/m^2 (OR 0.31, 95%CI: 0.17-0.55) compared to under- or healthy weight (BMI < 25 kg/m^2) as the reference. Similar results were seen in analyses adjusted for age and education. To determine whether the results were comparable for men and women, we stratified by sex. The results for women were similar to the overall results with lower risk for obese women. The results for men were null, but the number of men was small making estimates unstable with wide confidence intervals around risk estimates. Patients with microscopic colitis were more likely to report weight loss following the onset of diarrhea than controls: 65.3% cases *vs* 42.5% controls, p < 0.001. Because current BMI could be misclassified due to weight loss following the onset of diarrhea, we stratified on weight loss. The results were similar in the weight loss and the no weight loss strata. We conducted analyses separately for lymphocytic colitis and collagenous colitis and the results were similar to the overall.

All of the patients in our study had diarrhea. We asked patients if they had ever been told by a physician that they had irritable bowel syndrome (IBS). Not surprisingly the cases were less likely to have a history of IBS, odds ratio 0.40, 95%CI: 0.20 – 0.78). When we stratified by IBS, patients in the highest BMI category were less likely to have microscopic colitis in the non-IBS stratum, aOR 0.38 (95%CI: 0.18-0.79) an estimate similar to the overall estimate in cases and controls. We compared the BMI in patients with IBS stratified by microscopic colitis status. In the microscopic colitis cases, the mean BMI was not different in the IBS group (24.6 kg/m^2 (SD 7.2)) and the non-IBS group (25.7 kg/m^2 (SD 6.4), P = 0.55). Similarly, the mean BMI was not different among the controls with IBS (30.3 kg/m^2 (SD 6.6)) compared to non-IBS controls (29.1 kg/m^2 (SD 7.2), P = -0.27)).

Because the risk for microscopic colitis was lower in obese women than men (recognizing small numbers of men), we examined potential hormonal risk factors in women. Reproductive factors are shown in Table 3. The table shows crude odds ratios and odds ratios adjusted for age, education and BMI (model 1) and age, education, BMI, number of live births and age at menarche (model 2). Age at menarche, parity, number of live births and age at first live birth were not different in cases and controls. Use of oral contraceptive pills was inversely associated with microscopic colitis in crude (OR 0.41, 95%CI: 022-0.79), adjusted (aOR 0.38, 95%CI: 0.17-0.84), and multiply

Table 1 Characteristics of the study population

	Cases, *n* = 110		Controls, *n* = 252	
	n	Percent	*n*	Percent
Age[a]				
mean	63.2	12.7	54.5	11.8
Race[b]				
White	102	96.2	186	85.7
Non-White	4	3.8	31	14.3
Sex[a]				
Female	94	86.2	176	69.8
Male	15	13.8	76	30.2
Marital status				
Married	75	70.8	146	66.7
Not married	31	29.3	73	33.3
Education[a]				
Less than college	36	34.0	122	55.7
College or postgrad	70	66.0	97	44.3
Cigarette smoking[c]				
Never smoker	48	45.3	107	48.9
Former smoker	46	43.4	66	30.1
Current smoker	12	11.3	46	21.0
Irritable bowel syndrome[b]				
Yes	14	13.6	60	28.0
No	89	86.4	154	72.0

[a]$P < 0.001$.
[b]$P < 0.005$.
[c]$P < 0.05$.

adjusted analyses (aOR 0.20, 95%CI: 0.08-0.52). The results were the same when cases identified by pathology were excluded. The results were also similar for lymphocytic and collagenous colitis.

Menopausal factors are shown in Table 4. The percent of women who were postmenopausal was higher in the cases in crude analyses, but that is because they were older. The difference was absent in the adjusted model. Cases were more likely to have ever-used menopausal hormone hormones in the crude analysis OR 2.79, 95%CI: 1.44-5.41). After adjusting for age, education and BMI the risk estimate was lower (aOR 1.63, 95%CI: 0.73-3.62).

DISCUSSION

We found a striking difference in the risk for microscopic colitis with BMI, with a strong inverse association with obesity in women. Women with microscopic colitis were substantially less likely to be obese. There was no apparent effect of BMI on risk for microscopic colitis in men, although the number of men in our study was small. Given the sex differences, we also looked for possible reproductive or hormonal associations. We found a strong inverse association of microscopic colitis with ever use of oral contraceptives. In contrast to most prior studies, we enrolled patients who were referred for colonoscopy for diarrhea. At the time of the referral, the status as a microscopic colitis case or control was not known. All participants had similar symptoms, access to care, colonoscopy and biopsies.

Table 2 Body mass index and risk of microscopic colitis

	Cases		Controls			
	n = 101		*n* = 238		Crude	Adjusted[1]
BMI	*n*	%	*n*	%	OR (95%CI)	aOR (95%CI)
Overall						
< 25	50	49.5	73	30.7	Ref.	Ref.
25 < 30	29	28.7	60	25.2	0.71 (0.40-1.25)	0.70 (0.37-1.31)
≥ 30	22	21.8	105	44.1	0.31 (0.17-0.55)	0.34 (0.18-0.66)
Women						
< 25	47	53.4	47	28.7	Ref.	Ref.
25 -30	24	27.3	34	20.7	0.71 (0.36-1.37)	0.67 (0.32-1.40)
≥ 30	17	19.3	83	50.6	0.20 (0.10-0.40)	0.21 (0.10-0.45)
Men						
< 25	3	23.1	26	35.1	Ref.	Ref.
25 -30	5	38.5	26	35.1	1.67 (0.36-7.71)	2.22 (0.41-12.05)
≥ 30	5	38.5	22	29.7	1.97 (0.42-9.19)	2.92 (0.52-16.22)
Lost weight						
< 25	34	60.7	32	44.4	Ref.	Ref.
25 -30	13	23.2	17	23.6	0.72 (0.30-1.71)	0.89 (0.35-2.22)
≥ 30	9	16.1	23	31.9	0.37 (0.15-0.91)	0.49 (0.19-1.28)
No weight loss						
< 25	10	31.3	20	19.4	Ref.	Ref.
25 -30	13	40.6	30	29.1	0.87 (0.31-2.36)	0.64 (0.20-2.04)
≥ 30	9	28.1	53	51.5	0.33 (0.12-0.96)	0.33 (0.10-1.11)
IBS						
< 25	9	69.2	15	25.4	Ref.	Ref.
25-30	2	15.4	15	25.4	0.22 (0.04-1.21)	0.26 (0.04-1.50)
≥ 30	2	15.4	29	49.2	0.11 (0.02-0.60)	0.13 (0.02-0.76)
No IBS						
< 25	39	47.6	47	32.6	Ref.	Ref.
25 -30	26	31.7	37	25.7	0.85 (0.44-1.63)	0.78 (0.39-1.58)
≥ 30	17	20.7	60	41.7	0.34 (0.17-0.68)	0.38 (0.18-0.79)

[1]Adjusted for age and education. BMI: Body mass index; IBS: Irritable bowel syndrome.

Although BMI is an important risk factor for a number of diseases, there is surprisingly little information on the association between BMI and microscopic colitis. In a study using population controls, Larsen *et al*[17] reported data on 135 microscopic colitis who were compared to 27960 participants in the Malmo Diet and Cancer Study. There was no difference between the groups with respect to BMI. Similar to our study, Pascua *et al*[18] included 259 diarrhea controls in a small study with 26 microscopic colitis patients. There was no difference in BMI. Another small study was designed to examine the microbiome in 20 patients with microscopic colitis, 20 age- and sex-matched healthy controls, and 20 patients with functional diarrhea according to Rome IV criteria[19]. The BMI was 24.7 (SD 3.5) in microscopic colitis patients, 28.2 (SD 6.9) in healthy controls, and 27.9 (5.5) in the patients with chronic diarrhea. No statistics were reported in the paper, but based on the sample size and the estimates, the difference was significant for healthy controls (P = 0.05) and for diarrhea controls (P =

Table 3 Reproductive factors and risk of microscopic colitis in women

	Cases		Controls				
	n = 94		*n* = 176		Crude	Model 1[1]	Model 2[2]
	n	%	*n*	%	OR (95%CI)	aOR (95%CI)	aOR (95%CI)
Age of menarche							
≤ 11	18	20.5	46	30.3	Ref.	Ref.	Ref.
12	24	27.3	36	23.7	1.70 (0.80-3.61)	1.30 (0.53-3.17)	1.41 0.52-3.83)
13	23	26.1	38	25.0	1.55 (0.73-3.28)	0.76 (0.31-1.85)	0.54 (0.20-1.43)
≥ 14	23	26.1	32	21.1	1.84 (0.86-3.94)	0.90 (0.36-2.28)	0.85 (0.30-2.42)
Parity							
Nulliparous	15	16.5	29	19.0	Ref.	Ref.	Ref.[3]
Parous	76	83.5	124	81.1	1.04 (0.88-1.24)	1.02 (0.83-1.25)	1.00 (0.81-1.23)
Number of live births							
None	15	16.5	29	19.0	Ref.	Ref.	Ref.[3]
1	13	14.3	20	13.1	1.26 (0.49-3.20)	1.16 (0.39-3.48)	1.19 (0.40-3.60)
2	42	46.2	60	39.2	1.35 (0.65-2.83)	1.20 (0.50-2.89)	1.15 (0.47-2.81)
3 or more	21	23.1	44	28.8	0.92 (0.41-2.08)	0.78 (0.28-2.16)	0.83-0.30-2.35)
Age of first live birth (among parous women)							
≤ 19	13	17.1	32	25.8	Ref.	Ref.	Ref.
20-23	12	15.8	42	33.9	0.70 (0.28-1.75)	0.24 (0.07-0.82)	0.23 (0.07-0.85)
24-29	26	34.2	27	21.8	2.37 (1.02-5.49)	0.76 (0.24-2.43)	0.78 (0.23-2.63)
30+	25	32.9	23	18.6	2.68 (1.13-6.31)	1.07 (0.32-3.58)	1.02 (0.28-3.68)
Oral contraceptives							
No	27	29.7	23	15.0	Ref.	Ref.	Ref.
Yes	64	70.3	130	85.0	0.42 (0.22-0.79)	0.38 (0.17-0.84)	0.20 (0.08-0.52)

[1]Model 1 Adjusted for age, education and body mass index (BMI).
[2]Model 2 adjusted for age, education, BMI, age first live birth, number of live births, age of menarche.
[3]Omitted age of first live birth from model because of collinearity.

0.03). Roth *et al*[20] identified microscopic colitis cases from pathology records from 2002 – 2010 from the Skåne University Hospital, Malmö, with controls selected from a population-based study of breast cancer. The BMI in cases was 24.84 kg/m^2 and controls 24.88 kg/m^2, P = 0.451. The cases and controls were not recruited contemporaneously.

Cotter *et al*[13] sought to develop a scoring system to predict microscopic colitis among patients presenting with diarrhea. In a derivation cohort of 617 patients, BMI < 30 kg/m^2 was associated with an increased risk of microscopic colitis, OR 2.15 (95%CI: 1.19-3.88). Weight loss has been shown to be associated with MC in publications by the same authors[21,22], and it is not clear whether weight loss might have led to misclassification of BMI category. Liu *et al*[12] used the two Nurses' Health Study cohorts to identify 244 cases of self-reported microscopic colitis with 4.2 million person-years of observation. Compared to the women in the lowest BMI category, BMI < 18.5 kg/m^2, those with a BMI > 30 had an adjusted hazard ratio of 0.50 (95%CI: 0.32-0.79). The p for trend was < 0.001. Weight gain since early adulthood was also associated with reduced risk of microscopic colitis. The results were seen in both Nurses cohorts.

Cigarette smokers weigh, on average, 4–5 kg less than nonsmokers and are less likely to be overweight or obese[23]. Cigarette smoking has been associated linked with microscopic colitis in a meta-analysis[9]. In our study, however, current smoking was more common in the controls (who were heavier) and smoking was not associated with microscopic colitis after controlling for education.

Table 4 Menopausal factors and risk of microscopic colitis in women

	Cases		Controls			
	n = 94		*n* = 176		Crude	Adjusted
	n	%	*n*	%	OR (95%CI)	OR (95%CI)[1]
Menopausal status						
Pre-menopausal	13	14.4	40	26.1	Ref.	Ref.
Post-menopausal	77	85.6	113	73.9	2.10 (1.05-4.18)	1.20 (0.45-3.20)
Age at menopause						
< 45	17	24.3	49	45.8	0.37 (0.19-0.74)	0.64 (0.28-1.46)
45-54	43	61.4	46	43.0	Ref.	Ref.
55+	10	14.3	12	11.2	0.89 (0.35-2.27)	079 (0.28-2.22)
Menopausal type						
Surgical	31	40.3	70	62.0	Ref.	Ref.
Natural menopause	46	59.7	43	38.1	0.41 (0.23-0.75)	0.76 (0.37-1.54)
Menopausal hormones[2]						
No	46	60.5	90	81.1	Ref.	Ref.
Ever	30	39.5	21	18.9	2.80 (1.44-5.41)	1.63 (0.73-3.62)
Years Postmenopausal hormones[2]						
None	46	60.5	90	81.8	Ref.	Ref.
1-7 yr	15	19.7	11	10	2.67 (1.13-6.28)	1.65 (0.59-4.65)
9+ yr	15	19.7	9	8.2	3.26 (1.33-8.02)	1.68 (0.60-4.73)

[1]Adjusted for age, education and body mass index.
[2]Among women after menopause.

The controls in our study had diarrhea. If the diarrhea controls had a condition associated with obesity, that could potentially explain the findings. Many of the diarrhea patients who did not have microscopic colitis probably had irritable bowel syndrome. In a stratified analysis, the inverse association with obesity was particularly strong in the IBS strata (both cases and controls had a history of IBS). It is not clear that patients with IBS are more likely to be obese. A comprehensive review has not shown BMI differences in patients with irritable bowel syndrome[24]. In our study the controls with IBS did not have a higher BMI than the controls without IBS.

The mechanism by which increased body weight might be inversely associated with microscopic colitis in our study and others is uncertain. Men are at substantially reduced risk of microscopic colitis than women[4]. Perhaps the reduced risk is due to a protective effect of androgens. Obesity has been associated with increased levels of androgens[25,26]. Obesity has been linked with change in the gut microbiome[27] which has, in turn, been linked with microscopic colitis[19,28,29]. The gut microbiota can metabolize androgens and estrogens[30].

Despite the marked sex discrepancy in microscopic colitis, the literature on reproductive and hormonal factors is very limited. Burke *et al* used data from the Nurses' Health Study cohorts[31]. Similar to our results, the authors found no association with age of menarche, parity, age of first live birth or age of menopause. They found an increased risk for postmenopausal hormones. Verhaegh *et al*[32] reported that hormonal factors were not associated with microscopic colitis in multivariable analysis, although number of cases might not have been large enough to detect small differences between populations.

We found that ever-use of oral contraceptive pills was associated with a reduced risk of microscopic colitis in crude and adjusted analyses. In contrast to our study, Burke *et al*[31] reported that ever-use of oral contraceptives was associated with an increased risk of microscopic colitis based on data from the two Nurses' Health Study cohorts. There was a large difference in oral contraceptive use in the two cohorts - 45% in the Nurses' Health Study (NHS) and 85% in Nurses' Health Study II (NHSII). Oral

contraceptive use was not queried after 1984 in NHS (almost 40 years ago), or after 2009 in NHSII. The elevated risk was only statistically significant in the earlier cohort. There has been a temporal change in the hormone concentration of oral contraceptive pills, and the different results between our study and the Nurses' cohort study might be due to different times of enrollment. OCPs generally contain a mix of estrogen (usually estradiol) and a progestin. The estrogen dose has decreased from over 100 μg when first introduced in the 1960 to 20-30 μg or less now. Progestin-only contraceptives are also now available. These changes mean studies may not be comparable if they were performed at different times.

The mechanism behind the observed associations of menopausal hormones and oral contraceptives with microscopic colitis are unknown although there are some possibilities to consider. Estrogen and progesterone receptors are expressed in the gut, and sex steroids have been shown to influence colonic transit time, chloride secretion and intestinal permeability[33]. Autoimmune diseases are more common in women than men, possibly due to hormonal factors[34]. Hormonal contraceptives have effects on the immune system and have been linked with a number of different autoimmune diseases[35]. Microscopic colitis has been regarded as an autoimmune disease, perhaps because of similarities to celiac disease[36]. In a genome-wide association study using the UK Biobank, there was an association with single nucleotide polymorphisms on the MHC 8.1 haplotype, supporting an immune component to the pathogenesis of microscopic colitis[37]. We did not find autoimmune disease was over-represented in our microscopic colitis cases (unpublished data). The gut microbiome is involved in the metabolism, excretion and circulation of sex hormones[30]. The effect of menopausal hormones and oral contraceptives on microscopic colitis could be mediated by gut microbes. Gut microbes could metabolize sex steroids or exogenous steroids and alter the gut environment.

Our study had some important strengths. The patients were drawn from the same referral area and had similar access to and receipt of care. All of the patients had diarrhea. Other studies have used community[38], population[39], or disease controls [40]. A single experienced gastrointestinal pathologist reviewed all of the slides to classify patients as microscopic colitis cases or normal controls. The study included men and women. Detailed information was obtained from study participants using structured interviews.

A limitation of the study was the small size, particularly for men. Microscopic colitis is an uncommon disease and most reports in the literature are hampered by small numbers. Many of the patients referred for colonoscopy were either not eligible, cancelled their appointments or refused to participate. Nonresponse, along with the selected nature of the study population, may affect generalizability but should not lead to bias. Exposures were determined by self-report which is common in case-control studies. Recall of past exposures may be inaccurate, but we would not expect the recall for cases and controls to be differential as all of the patients were enrolled in the study because of diarrhea. . Cases were older than controls. We adjusted for age in all models and we performed sensitivity analyses with similar results. Lymphocytic colitis and collagenous colitis are considered to be histologic subtypes of the same disease[41] We combined the two entities to improve study power. In exploratory analyses we found similar results when we examine each type separately. The study was conducted in a developed country. Geographic variations in the incidence of microscopic colitis have been reported but there have been a limited number of direct comparative studies[1]. There are few studies from developing countries[42].

Microscopic colitis, first described in 1976, is a relatively new disease. With any new disease there is the presumption that an environmental factor, as opposed to a genetic factor, is responsible. Obesity has increased in the US since the 1980[43].The incidence of microscopic colitis has also been increasing in the US [44]. The fact that we and others have found lower risk of microscopic colitis with obesity suggests that obesity must interact with some other factor such as the microbiome to mediate risk. Given the difference in distribution in microscopic colitis by sex, exogenous hormones in the form of birth control pills and postmenopausal hormones are naturally of interest. In this study we found that postmenopausal hormones were modestly associated with a increased the risk of microscopic colitis and oral contraceptives with decreased risk.

As the population ages, the number of patients with microscopic colitis is likely to increase. Identifying factors associated with risk for microscopic colitis is an important first step developing hypotheses about etiology.

CONCLUSION

Compared to controls also seen for diarrhea, microscopic colitis cases were less likely to be obese. Mechanisms are unknown but could involve hormonal effects of obesity or the gut microbiome.

ARTICLE HIGHLIGHTS

Research background

There is limited information about risk factors for microscopic colitis, a leading cause of chronic watery diarrhea.

Research motivation

We hypothesized that obesity might be associated with microscopic colitis.

Research objectives

To compare patients with microscopic colitis to patients with chronic diarrhea to learn more about associations with obesity and hormones.

Research methods

We conducted a case-control study among patients who were referred to a single academic medical center for chronic diarrhea. The biopsies were reviewed by a research pathologist and classified as microscopic colitis cases or diarrhea controls. We used logistic regression to estimate odds ratios and 95% confidence intervals.

Research results

Cases with microscopic colitis had a lower body mass index than controls in adjusted models. Although patients with microscopic colitis reported that they lost more weight following the onset of diarrhea, the associations with BMI persisted in analyses stratified by weight loss. Oral contraceptives were inversely associated with microscopic colitis.

Research conclusions

Microscopic colitis cases were less likely to be obese than diarrhea controls. While the mechanism behind the association is not known, it could involve hormonal effects of obesity or the gut microbiome.

Research perspectives

Additional research is needed to understand the association between obesity and microscopic colitis.

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ORIGINAL ARTICLE

Observational Study

Identification of functional tumor necrosis factor-alpha promoter variants associated with *Helicobacter pylori* infection in the Sudanese population: Computational approach

Abeer Babiker Idris, Alaa B Idris, Manal A Gumaa, Mohammed Babiker Idris, Amanda Elgoraish, Mohamed Mansour, Dalia Allam, Bashir MO Arbab, Nazar Beirag, El-Amin M Ibrahim, Mohamed A Hassan

ORCID number: Abeer Babiker Idris 0000-0001-6436-5963; Alaa B Idris 0000-0002-2111-8070; Manal A Gumaa 0000-0002-3580-3327; Mohammed Babiker Idris 0000-0002-7316-8504; Amanda Elgoraish 0000-0003-2420-3120; Mohamed Mansour 0000-0002-1679-3071; Dalia Allam 0000-0002-7828-6091; Bashir MO Arbab 0000-0003-4555-9521; Nazar Beirag 0000-0003-2435-9969; El-Amin M Ibrahim 0000-0002-5175-0763; Mohamed A Hassan 0000-0003-3938-6898.

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Institutional review board

Abeer Babiker Idris, Department of Agricultural Science and Technology, Institute of Natural and Applied Sciences, Erciyes University, Kayseri 38039, Turkey

Abeer Babiker Idris, Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum 11111, Sudan. abeer.babiker89@gmail.com

Alaa B Idris, Department of Neurosurgery, Ribat University Hospital, Khartoum 11111, Sudan

Manal A Gumaa, El-Amin M Ibrahim, Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum 11111, Sudan

Mohammed Babiker Idris, BioMérieux Clinical and Application Advisor, Al-Jeel Medical Co., Riyadh 11422, Saudi Arabia

Amanda Elgoraish, Department of Epidemiology, Tropical Medicine Research Institute, Khartoum 11111, Sudan

Mohamed Mansour, Dalia Allam, Department of Gastroenterology, Ibn Sina Specialized Hospital, Khartoum 11111, Sudan

Bashir MO Arbab, Department of Gastroenterology, Modern Medical Centre, Khartoum 11111, Sudan

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Mohamed A Hassan, Department of Bioinformatics, Africa city of technology, Khartoum 11111, Sudan

Mohamed A Hassan, Department of Bioinformatics, DETAGEN Genetic Diagnostics Center, Kayseri 38350, Turkey

Mohamed A Hassan, Department of Translation Bioinformatics, Detavax Biotech, Kayseri 38350, Turkey

Corresponding author: Abeer Babiker Idris, DSc, Academic Research, Department of Agricultural Science and Technology, Institute of Natural and Applied Sciences, Erciyes

statement: The study was reviewed and approved by the Khartoum Ministry of Health research department, University of Khartoum, Faculty of Medical Laboratory Sciences review board, and Research Ethics Committees of hospitals.

Informed consent statement: Written informed consent was taken from participants before they enrolled in the study.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Data sharing statement: The data regarding TNF-A-1030 T>C genotypes and alleles distributions among participants and the in silico results of the software that used to support the findings of this study are available from the corresponding author at abeer.babiker89@gmail.com on a reasonable request.

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University, Merkez Kampüs Talas Yolu Melikgazi, Kayseri 38039, Turkey. abeer.babiker89@gmail.com

Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is a ubiquitous bacterium that affects nearly half of the world's population with a high morbidity and mortality rate. Polymorphisms within the tumor necrosis factor-alpha (*TNF-A*) promoter region are considered a possible genetic basis for this disease.

AIM

To functionally characterize the genetic variations in the *TNF-A* 5'-region (-584 to +107) of Sudanese patients infected with *H. pylori* using in silico tools.

METHODS

An observational study was carried out in major public and private hospitals in Khartoum state. A total of 122 gastric biopsies were taken from patients who had been referred for endoscopy. Genomic DNA was extracted. Genotyping of the *TNF-A*-1030 polymorphism was performed using PCR with confronting two-pair primer to investigate its association with the susceptibility to *H. pylori* infection in the Sudanese population. Furthermore, Sanger sequencing was applied to detect single nucleotide polymorphisms in the 5'-region (-584 to +107) of *TNF-A* in *H. pylori*-infected patients. Bioinformatics analyses were used to predict whether these mutations would alter transcription factor binding sites or composite regulatory elements in this region. A comparative profiling analysis was conducted in 11 species using the ECR browser and multiple-sequence local alignment and visualization search engine to investigate the possible conservation. Also, a multivariate logistic regression model was constructed to estimate odds ratios and their 95% confidence intervals for the association between *TNF-A* -1030, sociodemographic characteristics and *H. pylori* infection. Differences were statistically significant if $P < 0.05$. Statistical analyses were performed using Stata version 11 software.

RESULTS

A total of seven single nucleotide polymorphisms were observed in the *TNF-A* 5'-region of Sudanese patients infected with *H. pylori*. Only one of them (T > A, -76) was located at the in silico-predicted promoter region (-146 to +10), and it was predicted to alter transcription factor binding sites and composite regulatory elements. A novel mutation (A > T, +27) was detected in the 5' untranslated region, and it could affect the post-transcriptional regulatory pathways. Genotyping of *TNF-A*-1030 showed a lack of significant association between -1030T and susceptibility to *H. pylori* and gastric cancer in the studied population ($P = 0.1756$) and ($P = 0.8116$), respectively. However, a significant association was detected between T/C genotype and *H. pylori* infection (39.34% *vs* 19.67%, odds ratio = 2.69, 95% confidence interval: 1.17-6.17, $P = 0.020$). Mammalian conservation was observed for the (-146 to +10) region in chimpanzee (99.4%), rhesus monkey (95.6%), cow (91.8%), domesticated dog (89.3%), mouse (84.3%), rat (82.4%) and opossum (78%).

CONCLUSION

Computational analysis was a valuable method for understanding *TNF-A* gene expression patterns and guiding further *in vitro* and *in vivo* experimental validation.

Key Words: 5'-region; Promoter; TNF-A; *Helicobacter pylori*; In silico analysis; Sudan

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Core Tip: According to the literature, a considerable number of polymorphisms have

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been discovered in the tumor necrosis factor-alpha (*TNF-A*) promoter. A crucial question is whether these polymorphisms have any significant functional impact on disease incidence or severity. Seven single nucleotide polymorphisms were detected in the *TNF-A* 5'-region; only one of them, *TNF-A*-76, was located at the in silico-predicted promoter region (-146 to +10). This single nucleotide polymorphism may lead to the modification of the transcriptional regulation of *TNF-A* in *Helicobacter pylori* infection. Nevertheless, this conclusion cannot substitute for the experimental proofs, but it can provide direction or insight for them.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a widespread bacterium that affects nearly half of the world's population with a high morbidity and mortality rate[1]. Generally, the decreasing prevalence of *H. pylori* infection is continuing in many parts of North America and Europe. Still, no such decline has been noted in most developing countries, including Sudan, in which the *H. pylori* infection represents a critical public health challenge[2-4]. Although there is a common consensus that the risk of acquisition and transmission of *H. pylori* can be minimized and prevented to a large extent, this drop in those countries, whether it is a local phenomenon or a national trend, has to be investigated[2].

Infection with *H. pylori* is usually acquired during childhood. However, it may persist if not treated in the host's stomach throughout life due to the synchronized balance between the bacteria and its host[5,6]. The long duration of *H. pylori* in the gastric environment will develop chronic gastritis in *H. pylori*-infected persons. At the same time, a few may be complicated to more severe diseases such as peptic ulcer, atrophic gastritis, mucosa-associated lymphoid tissue lymphoma or gastric adenocarcinoma[7,8]. The variability in *H. pylori* infection outcomes is determined by bacterial factors, genetic characteristics of the host, especially those regarding polymorphisms in specific cytokines, and the environmental factors[7,9,10]. However, the crucial role of cytokine-related polymorphisms in the susceptibility or severity of *H. pylori* infection has been attracting considerable attention after El-Omar *et al*[11] proposed that functional polymorphisms in proinflammatory cytokine genes in *H. pylori*-infected patients are associated with a two- to three-fold increased gastric cancer risk. Because tumor necrosis factor-alpha (TNF-a) production has been implicated as an essential factor in immune regulations and inflammatory responses against *H. pylori* infection[12], polymorphisms within the *TNF-A* promoter are considered a possible genetic basis for this disease[13].

TNF-a is a potent pleiotropic proinflammatory cytokine that mediates diverse biological and pathological processes, in addition to its responsibility for the regulation of two opposite processes: proliferation and apoptosis (reviewed in[14,15]). *TNF* gene transcription is regulated by nucleoprotein complexes known as enhanceosomes that involve distinct sets of transcription factors and coactivators at the proximal *TNF* promoter and in the pattern of chromosomal organization of the *TNF/lymphotoxin* locus that function in synergy to drive transcription[16]. *TNF* enhanceosome assembly is cell type- and stimulus-specific, partially because of differential occupancy of overlapping DNA motifs in the *TNF* promoter. For example, after lipopolysaccharide stimulation of monocytes where no inducible translocation of NFATp can be detected, Sp1 and Ets/Elk proteins can successfully bind to sites that NFATp occupies in T cells[13,14]. Furthermore, many *TNF* activator binding motifs can be recognized by more than one class of transcription factor depending on the ambient concentration of different elements in the nucleus and the stimulus and cell type[16]. These features provide the *TNF* promoter with a remarkable degree of flexibility to respond to multiple stimuli in a specific manner through a short cis-

regulatory region[13].

The *TNF* locus lies within the major histocompatibility complex region on the short arm of human chromosome 6p21.3, a highly polymorphic region[17]. Many bi-allelic polymorphisms and microsatellites are found at or around the *TNF* locus[14,18]. Several have been suggested to be implicated in diseases susceptibility/resistance or severity by modifying the transcriptional regulation of *TNF*[13,18]. There are different transition variants in the *TNF-A* promoter region at the positions of -237, -307, -375, -856, -862 and -1030 in which the positions -307 and -237 have been most frequently evaluated for the association with *H. pylori* infection (susceptibility or progression)[13]. It has been reported that the presence of the adenine allele at -237 or -307 positions causes higher constitutive and inducible transcriptional levels than the guanine allele, which results in enhanced production of TNF-a up to five-fold *in vitro*[19,20]. In addition, the single nucleotide polymorphism (SNP) at the -1030 position, which is frequently observed in African populations[21,22], has been suggested to be related to high TNF-α production[23]. However, the influence of these SNPs on TNF-α production is not fully known and still a contradictory topic of debate, which is explained, in part, by ethnic differences and genetic variations between populations[19,20].

To our knowledge, there are no previous studies in Sudan that have addressed the association between *TNF-A* and *H. pylori* infection or *H. pylori*-associated diseases. Therefore, in this study, we applied DNA Sanger sequencing to detect genetic variations in the *TNF-A* 5′-region (-584 to +107) of Sudanese patients infected with *H. pylori* and predicted if these SNPs could alter transcription factor binding sites (TFBSs) or composite regulatory elements (CEs) using a computational approach. In addition, a comparative profiling analysis was performed to investigate the conservation of these SNPs in 11 species. Regulation mechanisms of TNF-α production may be linked to the difference in responses to *H. pylori* infection. This emphasizes the importance of studying the regulation of *TNF-A* gene expression.

MATERIALS AND METHODS

Research methodology

In the present study, in silico analysis was employed for the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* in two steps: (1) In silico prediction of the promoter region; and (2) In silico analysis of the predicted promoter region (-146 to +10). To obtain accurate results, we applied various software/servers to predict the promoter region, CpG island and regulatory motifs because promoters have complex and specific architecture and contain multiple transcription factors involved in particular transcription regulation[24]. Therefore, the unique composition of TFBSs and different features of each promoter may lead to different powers and algorithms for promoter identification[25]. In addition, genotyping of the *TNF-A*-1030 T>C polymorphism was performed to assess its association with *H. pylori* infection in the Sudanese population using PCR with confronting two-pair primer method.

However, the initial studies have numbered the positions of SNPs relative to the *TNF* mRNA cap site incorrectly (*e.g.*, -308 instead of -307 and -238 instead of -237)[13]. In contrast, the corrected numbering should have proceeded 5′ from the adenine at the +1 position[26] as in the Cytokine Gene Polymorphism In The Human Disease Database[27], and we used it throughout this study. The methodology followed in this study is represented in Figure 1.

Study population and study setting

An observational study was carried out in major public and private hospitals in Khartoum state in 2019-2020. These hospitals included Soba teaching hospital, Ibin Sina specialized hospital, Modern Medical Centre, Al Faisal Specialized Hospital and Police hospital. In addition, the molecular laboratory processes were conducted in the Molecular Biological Research laboratory at the Faculty of Medical Laboratory Sciences at the University of Khartoum.

The matched case-control formula was selected to calculate the sample size using Epi Info software version 7[28,29], assuming 95% confidence interval (CI), 80% power of the study, one ratio of control to the case, 15% of controls exposed, 3.36 odds ratio (OR) and 37.2% of cases exposed. According to the sample size calculation, the present study included 122 patients referred for upper gastrointestinal tract endoscopy, and most of them were because of dyspepsia. Out of that, 61 were *H. pylori*-negative patients and regarded as controls. The demographic characteristics of the study

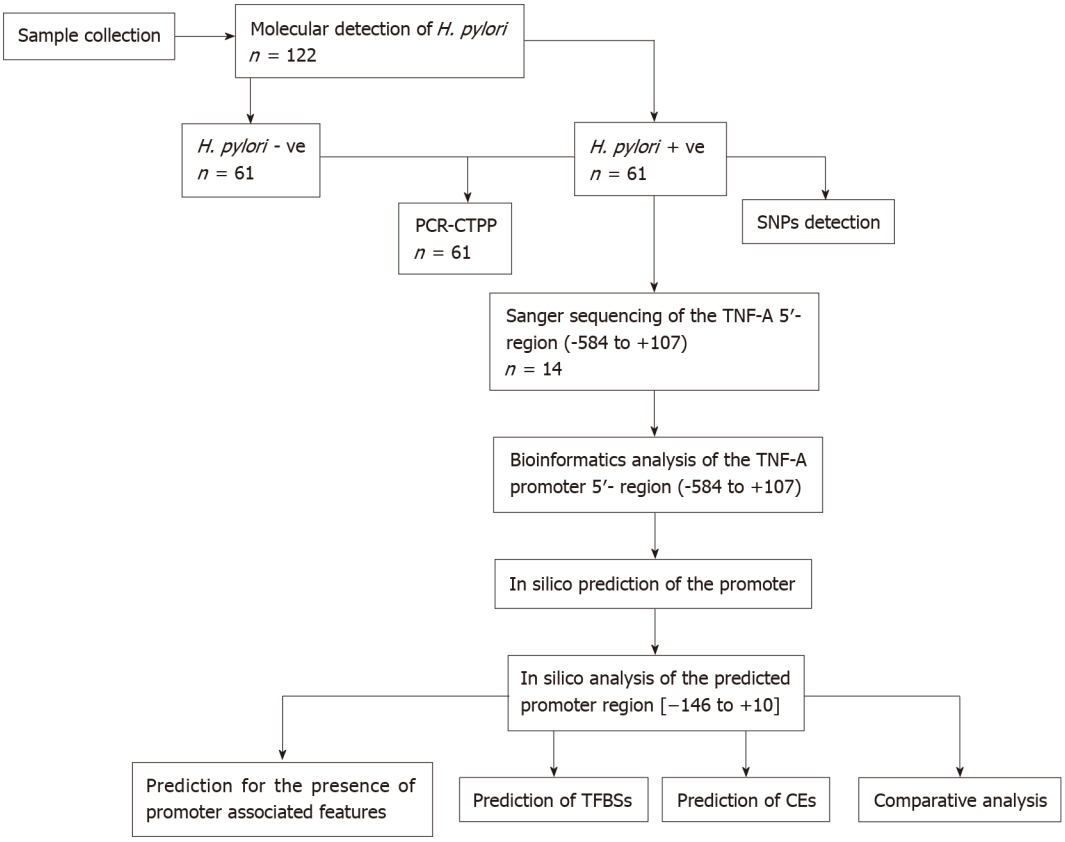


Figure 1 Schematic representation of the study methodology. *H. pylori*: *Helicobacter pylori*; -ve: Negative; +ve: Positive; CTPP: Confronting two-pair primer; SNP: Single nucleotide polymorphism; TNF-A: Tumor necrosis factor-alpha; TFBS: Transcription factor binding site; CE: Composite regulatory elements.

population are shown in Table 1.

The diagnosis of gastroduodenal diseases was based on the assessment of an experienced gastroenterologist, and in cancerous cases, histological examinations were also performed to confirm the diagnosis. The gastric pathologies of the study population are presented in Table 2.

The study population's demographic and clinical data were obtained by personal interviews, a structured questionnaire and a review of case records. The selection criteria included the Sudanese population from both sexes who were not taking antibiotics or nonsteroidal anti-inflammatory drugs. Written informed consent was granted by the participants after they were informed of the objectives and purposes of the study.

DNA extraction and PCR amplification of specific 16S rRNA gene of H. pylori

For DNA extraction purposes, gastric biopsies were collected in 400 μL of phosphate buffered saline. For histological investigation, the biopsies were preserved in 10% formalin. DNA extraction was performed by using innuPREP DNA Mini Kit (analytikjena AG, Germany) and followed the protocol that was given by the manufacturer, as previously described in[30].

Extracted DNA was amplified for the specific *16S* rRNA gene to diagnose and confirm the infection of *H. pylori* using the following primers (primers: F: 5′-GCGCAATCAGCGTCAGGTAATG-3′; and R: 5′-GCTAAGAGAGCAGCCTATGTCC-3′)[31] and the previously described PCR condition[4]. The amplified product for the *16S rRNA* is 522 bp.

Conventional PCR amplification and sequencing of the TNF-A promoter region

To amplify the promotor polymorphisms -308 and -238 of the *TNF-A* gene, primers: F: 5′-GCTTGTCCCTGCTACCCGC-3′ and R: 5′-GTCAGGGGATGTGGCGTCT-3′ were used[32]. The following thermal cycling conditions were used: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 60 s, 58 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The amplified PCR product is 691 bp located between -585 bp upstream and 107 bp downstream of the *TNF-A* gene.

Table 1 Demographic characteristics of the study population, *n* (%)

Variable		*Helicobacter pylori* (+ve)	*Helicobacter pylori* (-ve)	Total	*P* value
		n = 61	*n* = 61	*n* = 122	
Mean age in yr ± Standard deviation (range)		44.00 ± 16.99 (15-85)	44.74 ± 18.10 (17-89)	44.37 ± 17.48 (15-89)	0.9184[1]
Gender	Male	36 (50.00)	36 (50.00)	72 (59.02)	1.0000
	Female	25 (50.00)	25 (50.00)	50 (40.98)	
Residence	Urban	24 (44.44)	30 (55.56)	54 (44.26)	0.3622
	Rural	37 (54.41)	31 (45.59)	68 (55.74)	
Hospital	Public	21 (33.87)	31 (49.21)	52 (41.60)	0.0667
	Private	41 (58.57)	29 (41.43)	70 (57.38)	

[1]Mann-Whitney *U*, Gaussian Approximation. +ve: Positive; -ve: Negative.

Table 2 Gastric pathologies of the study population, *n* (%)

Endoscopy results	*n* (%)	*Helicobacter pylori* (+ve)	*Helicobacter pylori* (-ve)	*P* value[1]
Normal gastric findings	9 (7.40)	4 (6.45)	5 (7.94)	0.2686
Esophagitis	4 (3.30)	3 (4.80)	1 (1.59)	
Esophageal varices	6 (4.90)	1 (1.61)	5 (7.94)	
Gastritis	55 (45.10)	28 (45.16)	27 (42.86)	
Duodenitis	6 (4.90)	3 (4.84)	3 (4.76)	
Peptic ulcer	27 (22.13)	17 (27.42)	10 (15.87)	
Cancer	15 (12.30)	5 (8.20)	10 (16.4)	
Total	122 (100)	61 (50.00)	61 (50.00)	

[1]χ^2 test, df: 7.603, 6. +ve: Positive; -ve: Negative.

Fourteen PCR products of *H. pylori*-infected patients, which had the clearest bands, were sent for commercial DNA purification and Sanger dideoxy sequencing by Macrogen Inc., Korea.

Sequence analysis and SNPs detection

Two chromatograms (forward and reverse) for each sequence were visualized, checked for their quality and analyzed with the Finch TV program version 1.4.0[33]. In addition, the Basic Local Alignment Search Tool nucleotide (https://blast.ncbi.nlm.nih.gov/)was used to look for and assess nucleotide sequence similarities[34].

For SNP detection in the *TNF-A* promoter region, multiple alignment sequences were performed for the tested sequences with a reference sequence (*TNF-A*; NG_007462) using Clustal W[35].

Bioinformatics analysis of the TNF-A promoter region in H. pylori-infected patients

In silico prediction of the promoter: In this study, we employed five programs for human promoter prediction and recognition of human PolII promoter region and start of transcription (TSS) which include: Berkeley Drosophila Genome Project (http://www.fruitfly.org/)[36], Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/)[37], FPROM, TSSG and TSSW (http://softberry.com/) which are based on neutral network and linear discriminant approach[25,38,39].

In silico analysis of the predicted promoter region: Assessment for the presence of promoter-associated features: The computationally predicted promoter regions were additionally assessed for the presence of promoter-associated features using the ENCODE data (https://www.encodeproject.org/)[40-42]. These features include promoter-associated histone marks, DNase I hypersensitivity clusters, broad

chromatin state segmentation, transcription factor ChIP-seq and CpG islands.

Prediction of CpG islands: A CpG island is a DNA segment with high GC and CpG dinucleotide content and often regarded as a marker for the initiation of gene expression. In this study, we applied MethPrimer and GpC finder software (http://www.softberry.com/berry.phtml?topic=cpgfinder&group=programs&subgroup=promoter) to predict CpG islands using default search parameter values for the software: CpG island length >200 bp, CG% > 50%, and Obs/Exp > 0.6[43,44].

Prediction of TFBSs: Prediction of the potentially functional TFBSs is a crucial step in the chain of promoter analytical events. Therefore, we applied five prediction software programs to analyze the predicted promoter region for possible TFBSs: (1) AliBaBa2 (http://www.gene-regulation.com/)[45]; (2) Alggen Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/)[46,47]; (3) TF-Bind (http://tfbind.hgc.jp/)[48]; (4) Tfsitescan (http://www.ifti.org)[49]; and (5) Gene Promoter Miner (GPMiner) (http://GPMiner.mbc.nctu.edu.tw/), which is an integrated system. For predicting TFBSs, MATCH tool was utilized to scan TFBSs in an input sequence[50].

Prediction of CEs: CE is the minimal functional unit composed of two closely located DNA binding sites for distinct transcription factors, but its regulatory function is qualitatively different from the regulation effects of either individual DNA binding site. Thus, the MatrixCatch algorithm (http://gnaweb.helmholtz-hzi.de/cgi-bin/MCatch/MatrixCatch.pl) was used to identify the CEs in our region (-146 to +10)[51].

Comparative profiling analysis: ECR Browser (http://ecrbrowser.dcode.org)[52], NCBI Basic Local Alignment Search Tool nucleotide (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal W (https://www.genome.jp/tools-bin/clustalw)[35] were utilized to analyze the possible conservation of the predicted promoter region. The conservation was assessed in 11 species which include: Cow (*Bos taurus*), chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), dog (*Canis lupus familiaris*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), frog (*Xenopus laevis*), opossum (*Monodelphis domestica*), fugu pufferfish (*Takifugu rubripes*) and spotted green pufferfish (*Tetraodon nigrovoridis*). Furthermore, multiple-sequence local alignment and visualization search engine (https://mulan.dcode.org/) was used to evaluate the conservation of the promoter's SNPs and to screen the possible conservation of TFBSs at these SNP locations[53].

Molecular detection of the TNF-A-1030 C/T polymorphism using PCR with confronting two-pair primer method

Two sets of primers were used in PCR with confronting two-pair primer to detect the *TNF-A*-1030 C/T polymorphism as follow: for allele T, the sequences of the primers were F: 5'-AAGGCTCTGAAAGCCAGCTG-3' and R: 5'-CCAGACCCT-GACTTTTCCTTCA-3'; and for allele C, F: 5'-GAAGCAAAGGAGAAGCT-GAGAAGAC-3' and R: 5'-CTTCCATAGCCCTGGACATTCT-3'[54]. The PCR mixture components were the same as in[30]. The amplification conditions were initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 66 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The resulting PCR products were visualized by electrophoresis on a 1% agarose gel containing ethidium bromide. The predicted size was confirmed using a 50 bp DNA molecular weight marker. Genotyping was distinguished as follows; 444 bp and 316 bp for TT genotype, 444 bp, 316 bp and 174 bp for TC genotype and 444 bp and 174 bp for CC genotype.

Statistical analysis

Fisher's test or χ^2 test investigated Hardy-Weinberg equilibrium of allele and genotype distributions. Descriptive statistical analysis was applied to calculate percentages, proportions and means ± standard deviation. Regarding the incidence of *H. pylori* infection, differences in frequency distribution by age were examined by the Mann-Whitney test, while differences in the frequency distribution of the categorical demographic and clinical variables of the study population were assessed by Fisher's test or χ^2 test. A multivariate logistic regression model was constructed to estimate OR and their 95%CIs for the association between *TNF-A*-1030, sociodemographic characteristics and *H. pylori* infection. Differences were considered to be statistically significant if $P < 0.05$. The statistical analyses were performed using Stata version11 (StataCorp, College Station, Texas) software.

RESULTS

Nucleotide variations in the 5'-regulatory region (-585 to +107) of the TNF-A gene

In the Sudanese *H. pylori*-infected patients, a total of seven nucleotide variations were detected in the 5'-regulatory region (six substitution mutations and one insertion mutation of C at +70). Among which, five were bimodally mutated heterozygous SNPs, and two are homozygous SNPs, as illustrated in Table 3 and Figure 2. The results of the multiple sequence alignment were visualized in Jalview[55]. The nucleotide sequences of the *TNF-A* 5'-region (-584 to +107) were deposited in the GenBank database under the following accession numbers: from MW251712 to MW251725.

In silico prediction of the TNF-A promoter regions

Five types of promoter prediction programs were applied to predict the promoter regions of the *TNF* 5'-region (-584 to +107), and the results are overviewed in Table 4. Neural Network Promoter Prediction (NNPP version 2.2) predicted one promoter region, located at +1 bp relative to the TNF-α mRNA cap site[26]. In addition, FPROM and TSSG programs predicted two promoter regions -146 bp and +5 bp, respectively. The TSSW program also expected these two regions. In comparison, no promoter region was predicted by Promoter 2.0 Prediction Server.

In silico analysis of predicted TNF-A promoter region

Presence of promoter-associated features: ENCODE data revealed a high level of transcription factor occupancy patterns, promoter-associated histone modifications and DNase I hypersensitivity at the in silico predicted promoter region, from -146 to +10 nt relative to the TNF-α mRNA cap site[26]. Furthermore, CpGFinder and MethPrimer software were employed to predict the presence of CpG islands. However, both software predicted no CpG islands present in the predicted promoter region, and ENCODE data confirmed no presence of the CpG island in the predicted promoter (see Figure 3).

Conservancy of the predicted promoter: As illustrated in Figure 4, the ECR Browser showed mammalian conservation for the (-146 to +10) region in chimpanzee (*Pan troglodytes* - pan-Tro2) (99.4%), rhesus monkey (*Macaca mulatta* - rheMac2) (95.6%), cow (*Bos taurus* - bosTau3) (91.8%), domesticated dog (*Canis lupus familiaris* - canFam^2) (89.3%), rat (*Rattus norvegicus* - rn4) (82.4%), mouse (*Mus musculus* - mm9) (84.3%) and opossum (*Monodelphis domestica* - monDom5) (78.0%). But the region was not conserved in chicken (*Gallus gallus*), frog (*Xenopus laevis*), zebrafish (*Danio rerio*), fugu pufferfish (*Takifugu rubripes*) and spotted green pufferfish (*Tetraodon nigrovoridis*). An overview of the mammalian conservation of the in silico-predicted promoter of *TNF* is shown in Figure 5.

Prediction of TFBSs: In the present study, we used five software programs to predict TFBSs. To ensure accurate analysis, only factors predicted by three out of the five programs or predicted by two programs but verified in the literature were selected. The five prediction programs reported multiple putative TFBSs within the (-146 to +10) region, as illustrated in Table 5. Moreover, screening of this region using the NCBI SNP databases showed four SNPs, as shown in Table 5. The ECR Browser and NCBI Basic Local Alignment Search Tool nucleotide revealed the conservation of these SNPs in chimpanzees, rhesus monkey, cow and dog. Multiple-sequence local alignment and visualization presented multiple TFBSs to be located at rs765073823 and rs537401710. The overview from the multiple-sequence local alignment and visualization software for conserved TFBSs predicted to be conserved (100%) between humans, chimpanzees, rhesus monkeys, cows, and domesticated dogs is summarized in Table 6.

Prediction of CEs: In the present study, CEs were predicted using MatrixCatch, which can find experimentally known regulatory elements (both single sites and pairs) and novel regulatory elements by computational comparison to the known ones in a library of CE models[51]. An overview of predicted CEs by MatrixCatch is presented in Table 7.

Allele and genotype frequencies of TNF-A-1030 and susceptibility to H. pylori infection

Sixty-one participants from each group (*H. pylori*-infected and uninfected) were successfully genotyped for *TNF-A*-1030 polymorphism; a representative gel is shown

Table 3 Nucleotide variations in the 5'-regulatory (-584 to +107) region of tumor necrosis alpha in Sudanese *Helicobacter pylori*-infected patients

SNP	Event	SNP locus[1]	Chromosomeposition	Serialnumber (rs)
C>G (homozygous)	Transversion	-567	NG_007462.1:g.4422 C>G	rs2857711
G>A (heterozygous)	Transition	-375	NG_007462.1:g.4614 G>A	rs1800750
G>A (heterozygous and homozygous)	Transition	-307	NG_007462.1:g.4682 G>A	rs1800629
G>A (heterozygous)	Transition	-237	NG_007462.1:g.4752 G>A	rs361525
T>A (heterozygous)	Transversion	-76	NG_007462.1:g.4913 T>A	rs41297589
A>T (heterozygous)	Transversion	+27	NG_007462.1:g.5016 A>T[2]	-
Insertion of C	-	+70	NG_007462.1:g.5058-5059 InsC	-

[1]Position of the single nucleotide polymorphism is relative to the tumor necrosis factor-alpha mRNA cap site[26].
[2]Novel mutation at the 5′ untranslated region detected in this study. SNP: Single nucleotide polymorphism.

Table 4 Overview of the in silico predicted tumor necrosis factor-alpha promoter regions for the respective prediction programs. The most predicted region is indicated in bold

Prediction program	Predicted promoter regions[1]
BDGP (NNPP version 2.2)[2]	-40 to +10
Promoter 2.0 Prediction Server	no promoter
FPROM	+5
TSSG[3]	-146
TSSW[4]	-140
	+6

[1]All positions are relative to the tumor necrosis factor-alpha mRNA cap site[26].
[2]NNPP: Neural network promoter prediction; Threshold 0.80.
[3]Threshold for LDF- 4.00.
[4]Thresholds for TATA+ promoters - 0.45, for TATA−/enhancers - 3.70.

in Figure 6. Allele and genotype frequencies of *TNF-A*-1030 polymorphism in patients infected with *H. pylori* and uninfected controls are presented in Table 8. Although the TNF-*A*-1030-C allele was more prevalent in patients infected with *H. pylori* compared to uninfected controls, no significant association was found between the allele frequencies of *TNF-A*-1030 and *H. pylori* infection (27.87% *vs* 19.67%, OR = 0.6339, 95%CI: 0.3490-1.151, P = 0.176). However, a significant association was detected between T/C genotype and *H. pylori* infection (39.34% *vs* 19.67%, OR = 2.69, 95%CI: 1.17-6.17, P = 0.020) (Tables 8 and 9). In contrast, no significant association was observed between allele and genotype frequencies of *TNF-A*-1030 and gastric cancer (P = 0.8116 and P = 0.3900, respectively) (Table 10). The allelic and genotype distributions at -1030 of *TNF-A* were in Hardy-Weinberg equilibrium, with non-significant χ^2 values (for uninfected subjects P = 0.1520; and subjects with benign disorders P = 0.2089).

DISCUSSION

Many SNPs have been observed in the vicinity of the *TNF* locus, particularly in the distal *TNF* promoter. Some of these have been implicated in the disease susceptibility, resistance or severity by modifying the transcriptional regulation of *TNF*[13,56-58]. In this study, we functionally analyzed SNPs in the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* and had divergent clinical outcomes that ranged from simple asymptomatic gastritis to more serious conditions, *i.e.* peptic ulcer disease and gastric cancer. The exploratory sequencing of *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* revealed six previously reported

Table 5 Summary of the in silico predicted transcription factor binding sites for tumor necrosis factor-alpha (-146 to +10) region

TranscriptionFactor	Position[1]		SNP in binding site	Software				
	Start	End		AliBaba2.1	Alggen Promo	Tfsitescan	TF-Binding	GPMiner
Elk-1	-145	-130			X[2]		X	
Sp1	-138	-126		X		X		
AP-2	-129	-118			X		X	
Ets-1	-118	-111				X		
PEA3/ Ets-1	-118	-110				X		
T3R_TRE1	-115	-108			X	X	X	
TNF-alpha-CRE	-112	92				X	X	
C/EBP-beta	-100	-74	rs41297589 [-76][3]			X	X	
NF-kappaB	-99	-86				X	X	
AP-1	-67	-57				X		
NF-kappaB	-55	-46	rs765073823 [-48]rs537401710 [-47]	X				
Sp1	-54	-41	rs765073823 [-48]rs537401710 [-47]	X	X	X		
Sp1	-48	-39	rs765073823 [-48]rs537401710 [-47]	X	X	X		
GATA	-40	-30	rs539666421 [-38]	X	X		X	
AP-2	-38	-27	rs539666421 [-38]	X		X	X	
TBP	-29	-20		X			X	X

[1]Position is relative to the tumor necrosis factor-alpha transcription start site.
[2]X means the transcription factor is predicted by the software that is marked with X.
[3]Observed in this study. SNP: Single nucleotide polymorphism.

Table 6 Overview of conserved transcription factor binding sites predicted by multiple-sequence local alignment and visualization

Transcription Factor	Binding sequence	Position[1]		Conserved SNPs
		Start	End	
V$CETS168_Q6	gCTTCCTC	-115	-108	
V$ETS_Q6	gcTTCCtc	-115	-108	
V$SP1_Q4_01	ttccCCGCCCtcc	-51	-39	rs765073823 [-48]rs537401710 [-47]
V$SP1_Q6_01	tcCCCGCCCt	-50	-41	rs765073823 [-48]rs537401710 [-47]
V$SP1_Q2_01	cCCCGCCCtc	-49	-40	rs765073823 [-48]rs537401710 [-47]

[1]All positions are relative to the tumor necrosis factor-alpha mRNA cap site. Multiple-sequence local alignment and visualization (https://mulan.dcode.org/) to be conserved (100%) between human, chimpanzee, rhesus monkey, cow, and domesticated dog at the (-146 to +10) region. The crucial transcription factor for tumor necrosis factor transcription, nuclear factor, was not included.

SNPs located at -567, -375, -307, -237, -76 and +70 and one novel SNP at +27 relative to the *TNF-A* transcription start site. A C to G homozygous transversion at -567 nt was found in all our studied populations. At the same time, the novel A to T heterozygous transversion at +27 nt was observed in five patients who had normal upper gastro-endoscopy or gastritis. This SNP is located in the 5′ untranslated region. Therefore, it could affect the post-transcriptional regulatory pathways that control mRNA localization, stability and translation efficiency[59]. Further studies are encouraged to investigate this SNP in terms of the frequency of the minor allele (T) in the Sudanese population and its functional significance using computational and experimental

Table 7 Summary of the in silico predicted composite regulatory elements for the -146 to +10 region of tumor necrosis factor-alpha

Composite element	MatrixName for a 1st element	Score, strand	Distance in between	MatrixName for a 2nd element	Score, strand	Position of CE, orientation	orientation	Composite score	*P* value	Sequence
CE00266	V$ETS_Q6	0.991+	12	V$AP1_Q2_01	0.831+	-118	+	0	1.08e-03	GCTTCCTCCagaTGAGCTCATGGG
CE00109	V$AP1_C	0.853-	8	V$NFAT_Q6	0.943-	-66	-	0.007	3.42e-03	TTGAATGATTCTTTCCCCGC
CE00150	V$AP1_C	0.853-	8	V$NFAT_Q6	0.943-	-66	-	-0.237	3.42e-03	TTGAATGATTCTTTCCCCGC
CE00058	V$HMGIY_Q6	0.955-	-5	V$NFKB_Q6_01	0.759-	**-78**	-	-0.09	6.89e-03	GTTTTCCGCTG
CE00058	V$HMGIY_Q6	0.955-	-4	V$NFKB_Q6_01	0.662-	**-78**	-	0.005	9.20e-03	GTTTTCCGCTGG
CE00064	V$HNF3_Q6	0.780+	15	V$NF1_Q6	0.956+	-28	+	-0.254	4.83e-02	ACATATAAAGGCAGtTGTTGGCACACC

CE: Composite regulatory elements.

Table 8 Allele and genotype frequencies of tumor necrosis factor-alpha-1030 polymorphism among *Helicobacter pylori*-infected and uninfected subjects and their contributions to *Helicobacter pylori* infection, *n* (%)

	H. pylori (+ve), *n* = 61	*H. pylori* (-ve), *n* = 61	*P* value	OR (95%CI)
Allele frequency				
TNF-A-1030-T	88 (72.13)	98 (80.33)	0.176	0.63 (0.349-1.151)
TNF-A-1030-C	34 (27.87)	24 (19.67)		
Genotype frequency				
T/T	32 (52.46)	43 (70.49)	-	1 (reference)
T/C	24 (39.34)	12 (19.67)	0.020[a]	2.69 (1.17-6.17)
C/C	5 (8.20)	6 (9.84)	0.862	1.12 (0.31-3.99)

[a]*P* value < 0.05 statistically significant difference. OR: Odds ratio; 95%CI: 95% confidence interval; *H. pylori*: *Helicobacter pylori*; +ve: Positive; -ve: Negative; *TNF-A: Tumor necrosis factor-alpha*.

approaches.

Intriguingly, the heterozygous SNP T>A at -76 was located at the in silico-predicted promoter region. However, in this study, the in silico analysis predicted promoter region was located from -146 to +10 nt relative to the TNF-α mRNA cap site using different algorithms[26]. This finding is in agreement with previous studies conducted on 411 *TNF* promoters from individuals of distinct ethnic backgrounds that concluded that the human *TNF* promoter sequences up to 200 bp relative to the transcription start site involved in enhanceosome formation and the regulation of the gene are completely conserved in humans[21,26,60,61]. Also, the ENCODE data showed a high level of the hallmarks of cis-regulatory regions at the in silico predicted promoter region (-146_+10), which include nuclease hypersensitivity and histone modifications [40-42].

Functional elements in the mammalian gene promoters can be detected by high conservation between species distantly related on the evolutionary scale[62,63]. In this study, the computational comparative analysis revealed mammalian conservation for the -146 to +10 region in chimpanzee (*Pan troglodytes* - pan-Tro2) (99.9%), rhesus monkey (*Macaca mulatta* - rheMac2) (93.7%), cow (*Bos taurus* - bosTau3) (78.2%), domesticated dog (*Canis lupus familiaris* - canFam[2]) (77.8%), rat (*Rattus norvegicus* - rn4) (76.3%), mouse (*Mus musculus* - mm9) (78.7%) and opossum (*Monodelphis domestica* - monDom5) (72.2%). In an earlier study conducted by Leung *et al*[64], the region from positions -131 to -63 and -53 and -45 relative to the start site of *TNF* gene transcription showed high sequence conservation among primates, with complete conservation

Table 9 Multivariate analysis of genotype frequencies of tumor necrosis factor-alpha-1030 polymorphism and sociodemographic characteristics in *Helicobacter pylori* infection

Variables		*P* value	OR (95%CI)
Age		0.946	0.99 (0.98-1.02)
Gender	Male		1 (reference)
	Female	0.845	0.93 (0.42-2.02)
Residence	Urban		1 (reference)
	Rural	0.341	1.46 (0.67-3.18)
Hospital	Private		1 (reference)
	Public	0.045[1]	0.45 (0.21-0.98)
Genotype frequency	T/T		1 (reference)
	T/C	0.016[a]	2.87 (1.22-6.78)
	C/C	0.982	1.02 (0.27-3.88)

[1]Weak evidence of significant different.
[a]*P* value < 0.05 statistically significant different. OR: Odds ratio; 95%CI: 95% confidence interval.

Table 10 *Tumor necrosis factor-alpha*-1030 polymorphism and gastric cancer association risk in gastric cancerous patients and subjects with benign gastric disorders, *n* (%)

	Gastric pathologies		OR (95%CI)
	Benign disorders[1], *n* = 108	Cancer, *n* = 14	
Allele frequency			
TNF-A-1030-T	176 (90.28)	21 (75.00)	1.150 (0.4627 - 2.8600)
TNF-A-1030-C	51 (9.72)	7 (25.00)	
P value	0.8116		
Genotype frequency			
T/T	68 (62.96)	7 (50.00)	1.700 (0.5556 - 5.2020)
C carrier	40 (37.04)	7 (50.00)	
P value	0.3900		

[1]Benign disorders, *i.e.* gastritis, duodenitis or ulcer.
OR: Odds ratio; 95%CI: 95% confidence interval; *TNF-A: Tumor necrosis factor-alpha*.

within phylogenetic footprints across seven primate species, including Old World monkeys and New World monkeys[64].

However, the *TNF-A* gene is located within the major histocompatibility complex region of chromosome 6, the highly polymorphic region of the human genome, which includes the human leukocyte antigen (HLA) genes[65]. In fact, all of the common ten detected SNPs (-1030, -862, -856, -574, -375, -307, -243, -237, and +70 nt relative to the start site of transcription) fall outside of the identified *TNF* primate phylogenetic footprints[66-70]. This feature is consistent with the accrual of mutations under neutral evolution[64,72]. Furthermore, several of these SNPs (-1030, -862, -856, -307, -243, -237) have been shown to be in non-random association with extended HLA haplotypes[21,26,71-73], which are HLA regions that exhibit fixity of the genomic interval between HLA-B and HLA-DR, that includes *TNF-A* and complement (complotype) regions[21,74,75].

Intriguingly, we detected one mutation (T>A, -76) located at the identified *TNF* primate phylogenetic footprints[64] in a patient with reinfection with *H. pylori* multiple times. The presence of this SNP in the conserved region that is involved in regulating the *TNF* gene could affect the expression and create a condition of hypoacidity that favors the survival and colonization of *H. pylori*[76,77]. The -76 SNP

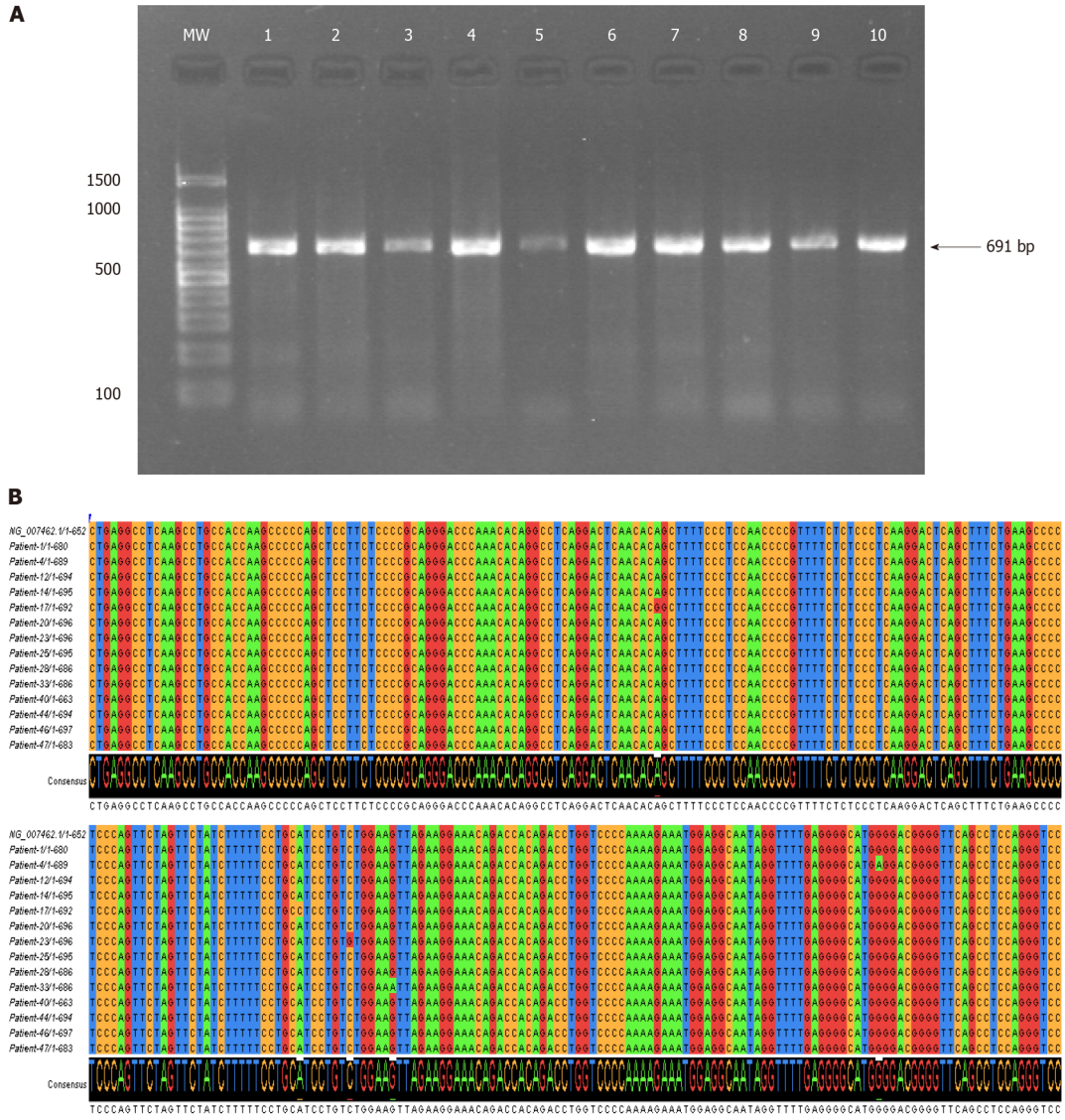


Figure 2 Nucleotide variations of the tumor necrosis factor-alpha 5'-region (-584 to +107) in the Sudanese *Helicobacter pylori*-infected patients. A: PCR amplification results of the tumor necrosis factor-alpha 5'-region (-584 to +107) examined on 1% agarose gel electrophoresis; B: Multiple sequence alignment of the nucleotide sequences of the tumor necrosis factor-alpha 5'-region performed in Clustal W and visualized in Jalview[55]. The levels of identity are visible as histograms at the bottom.

was first discovered with an overall minor allele frequency of 0.031 in the Indian population while studying polymorphisms of *TNF*-enhancer and the gene for FcγRIIa correlated with the severity of *Falciparum malaria* in the ethnically diverse Indian population[78]. It is present at the low-affinity transcription factor Ets-1 binding site in macrophages and the high affinity NFATp binding site in T and B cells[79]. Also, it is located at an in silico-predicted C/EBP-beta (also known as NF-IL6) binding site (in this study, see Table 5), which was experimentally proven in myelomonocytic cells[80-82].

Sequence analysis of the *TNF-A* 5'-region (-584 to +107) of the Sudanese patients revealed relative variability of *TNF-A* SNPs (Figure 2 and Table 3). This finding is in agreement with a study conducted by Baena *et al*[21] that found that the highest relative diversity of *TNF-A* SNPs was revealed among the three African studied populations (Malawian, Southern Nigerian, African-American and African-Caribbean). Also, it is consistent with the "out of Africa" hypothesis based upon human mitochondrial DNA analyses[83]. Despite this high level of variation, here in this study, the sequences involved in enhanceosome formation and gene regulation are highly conserved[64]. Remarkably, we detected only a single SNP (-76) in this region. By contrast, the novel +27 variant and other *TNF-A* SNPs described in this study occur within or immediately adjacent to promoter regions with high diversity in the primate lineage. Unexpectedly, the -243 variant, the first *TNF-A* SNP linked to an African-derived extended HLA haplotype[70], was not detected in our studied population.

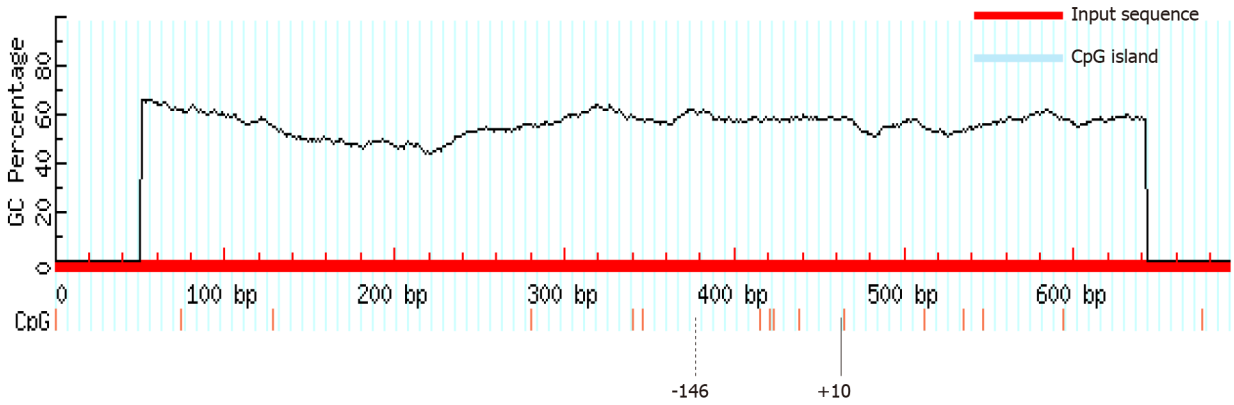


Figure 3 MethPrimer software prediction of no CpG islands in the in silico predicted promoter region.

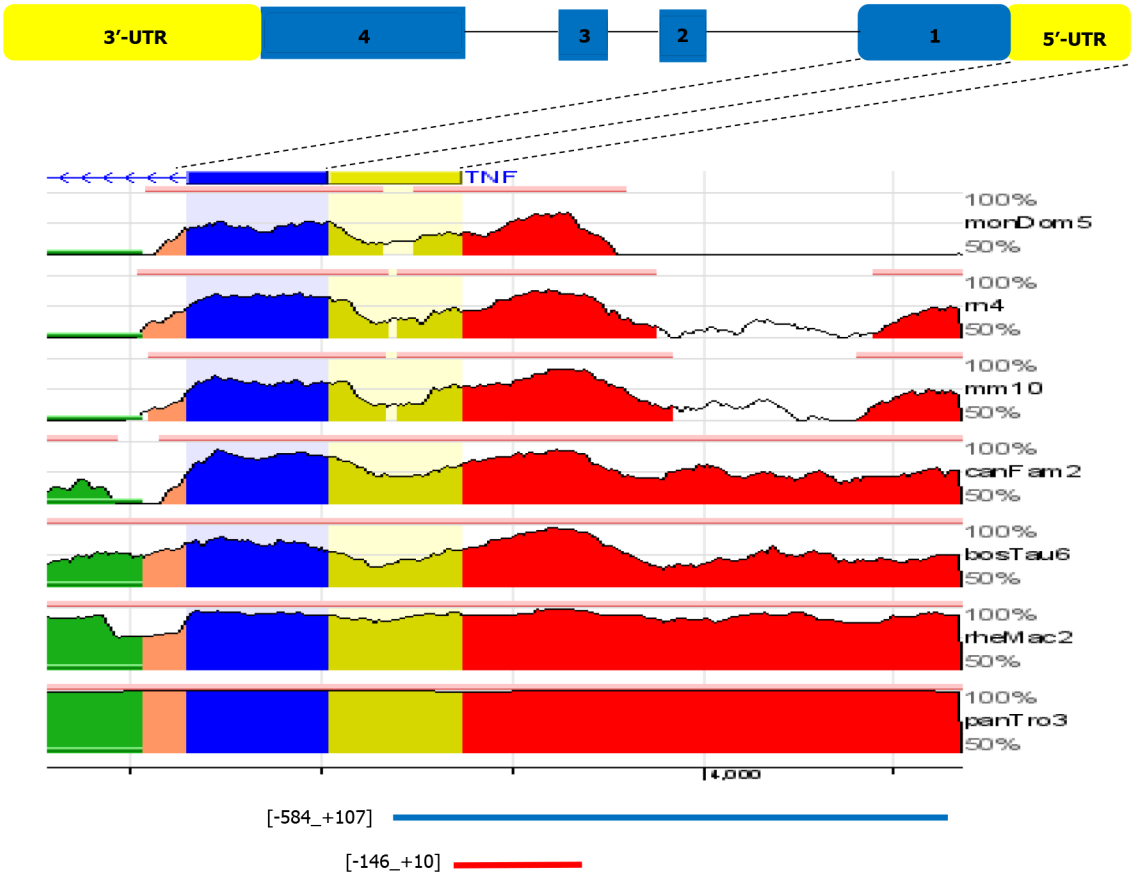


Figure 4 Overview from the ECR Browser shows mammalian conservation of the tumor necrosis factor 5'-upstream region compared to the human sequence in the region (-584 to +107) (hg19 chr6:31542751-31543444). Blue boxes represent the first tumor necrosis factor exon, while yellow indicates the tumor necrosis factor 5' untranslated region. Intragenic positions are highlighted in red or in green when corresponding to transposable elements and simple repeats. UTR: Untranslated region.

Other previously identified SNPs in this region (-584 to +107), which include -567, 375, -307, -237, -76 and +70 nt relative to the start site of transcription, were observed[66-68, 84].

The -1030 (T/C; rs1799964) SNP is frequently observed in African populations (17%-25% Malawian, 7% Nigerian, 29% African-American/Caribbean and 14% Gambia)[21, 22]. In this study, the -1030 SNP was detected in 47 Sudanese (38% of our studied population). Many studies have been published that examine the association between the -1030 SNP and multiple diseases[60,85,86] and diseases related to *H. pylori* such as gastroesophageal reflux disease, gastritis, peptic ulceration and gastric cancer[87-90]. In this study, we found a lack of association between -1030T and susceptibility to *H.*

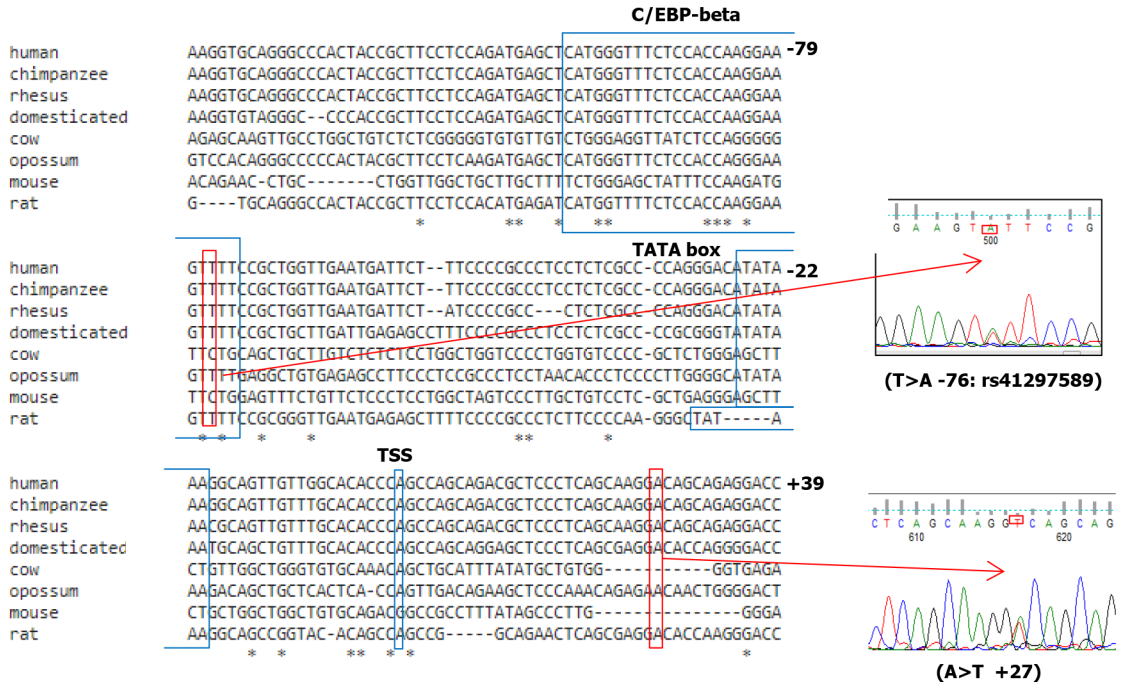


Figure 5 Mammalian conservation of (AT; single nucleotide polymorphism databases dbSNP: rs41297589) at position -76 and the novel mutation (A>T at +27) in the 5' untranslated region among various species. The nucleotides are enumerated at each line on the right side, and the in silico predicted TATA-, C/EBP-beta and transcription start site have marked inboxes. The chromatogram results of the polymorphisms are visualized using Finch TV software. TSS: Transcription start site.

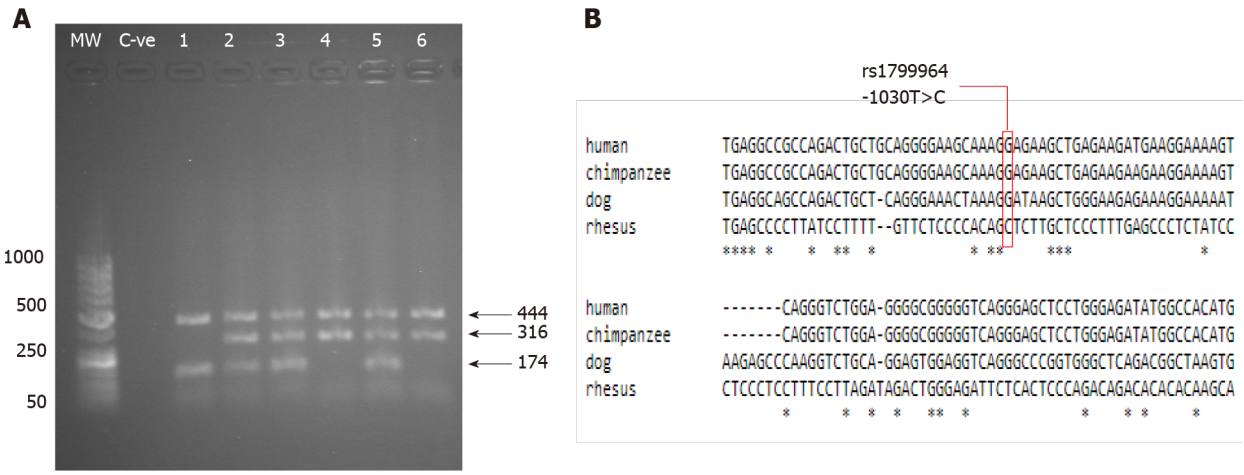


Figure 6 Molecular detection of the tumor necrosis factor-alpha-1030 C/T polymorphism. A: PCR with confronting two-pair primer products analyzed on a 1% agarose gel stained with ethidium bromide. Three genotypes can be seen. Lanes 2, 3 and 5 showed 444 bp, 316 bp and 174 bp, indicating a heterozygous genotype. Lanes 4 and 6 showed 444 bp and 316 bp, which indicated a homozygous T genotype. Lane 1 showed 444 bp and 174 bp, which indicated a homozygous C genotype; B: Mammalian conservation of (TC; dbSNP: rs1799964) position -1030 among different species. MW: Molecular weight marker; C-ve: Negative control.

pylori and gastric cancer in the studied population (P = 0.1756 and P = 0.8116, respectively). This finding is in agreement with previous studies[87,88,91]. In contrast to a study conducted in Japan that observed a significant association between -1030T and gastric cancer[91]. This variation could be attributed mainly to differences in genetic backgrounds of the studied population, the method of genotyping and sample size[76,92].

The study's limitations include the relatively small number of the enrolled subjects and depending on the in silico tools for studying the influence of the detected promoter variants on the *TNF-A* gene expression and *H. pylori* infection (susceptibility and progression). Further large cohort studies are needed to validate the study findings. However, the computational analysis provides a basis for identifying

promoter regions, recognizing regulatory motifs and understanding gene expression patterns[25].

According to the literature, a considerable number of polymorphisms have been discovered in the *TNF-A* promoter. However, a crucial question that remains to be answered is whether these polymorphisms have any functional effect that may significantly impact disease incidence or severity. Identifying which of these many variants are functional is of great significance for discovering new preventive, diagnostic and therapeutic strategies against the incidence and/or progression of multifactorial diseases such as *H. pylori* infection. This study detected seven SNPs in the *TNF-A* 5′-region; only one of them (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10). This SNP could lead to the modification of the transcriptional regulation of *TNF-A* in *H. pylori* infection. However, this conclusion cannot substitute for the experimental proofs (*in vitro* or *in vivo*), but it can provide a direction or insight for such experiments to validate the in silico predictions.

CONCLUSION

In the Sudanese *H. pylori*-infected patients, seven SNPs were observed in the *TNF-A* 5′-region; only one of them (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10). In addition, it was predicted to alter TFBSs and CEs. Furthermore, a novel mutation (A>T, +27) was detected in the 5′ untranslated region, and it could affect the post-transcriptional regulatory pathways. In addition, computational analysis was a valuable method for understanding gene expression patterns and providing guidance for further *in vitro* and *in vivo* experimental validation.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) infection represents a major public health challenge in Sudan. However, functional polymorphisms within the tumor necrosis factor-alpha (*TNF-A*) promoter are associated with the incidence and progression of *H. pylori* infection by increasing TNF-α production.

Research motivation

TNF lies within the major histocompatibility complex region of chromosome 6, which is a highly polymorphic region. Therefore, many polymorphisms have been detected in the *TNF-A* promoter and studying which variants could affect *TNF-A* gene expression is relevant. However, the influence of these single nucleotide polymorphisms (SNPs) on TNF-α production is not fully known and still a contradictory topic of debate due to the ethnic differences between populations. Furthermore, to our knowledge, there are no previous studies in Sudan that have addressed the association between *TNF-A* and *H. pylori* infection or *H. pylori*-associated diseases.

Research objectives

To functionally characterize the genetic variations in the *TNF-A* 5′-region (-584 to +107) of Sudanese patients infected with *H. pylori* and predict if these SNPs could alter the regulatory motifs using bioinformatics analyses. Also, to investigate the mammalian conservation of these SNPs using comparative profiling analysis in 11 species.

Research methods

An observational study was conducted in the major hospitals in Khartoum state. Genomic DNA was extracted from 122 gastric biopsies of patients who had been referred for endoscopy. Genotyping of the *TNF-A*-1030 polymorphism was performed using PCR with confronting two-pair primer to investigate its association with *H. pylori* infection in the Sudanese population. Sanger sequencing was applied to detect SNPs in the 5′-region (-584 to +107) of *TNF-A* in *H. pylori*-infected patients; in silico tools were used to predict whether these mutations would alter transcription factor binding sites or composite regulatory elements in this region. In addition, the ECR browser and multiple-sequence local alignment and visualization search engine were

used to study the conservation of the detected SNPs among 11 mammalian species.

Research results

A total of seven SNPs were observed in the *TNF-A* 5′-region of Sudanese patients infected with *H. pylori*. Among them, the SNP (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10), and it was predicted to alter transcription factor binding sites and composite regulatory elements, while the novel mutation (A>T, +27) was detected in the 5′ untranslated region. It could affect the post-transcriptional regulatory pathways. Mammalian conservation was detected for the (-146 to +10) region in chimpanzee (99.4%), rhesus monkey (95.6%), cow (91.8%), domesticated dog (89.3%), mouse (84.3%), rat (82.4%) and opossum (78.0%). Furthermore, genotyping of *TNF-A*-1030 revealed a lack of significant association between -1030T and susceptibility to *H. pylori* and gastric cancer in the studied population (P = 0.1756 and P = 0.8116, respectively).

Research conclusions

Despite the high level of genetic variation in the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients, the sequences involved in enhanceosome formation and gene regulation are highly conserved. Remarkably, only a single SNP (-76) was detected in this region. In addition, computational analysis was a valuable method for studying gene expression patterns and insights for further *in vitro* and *in vivo* experimental proofs.

Research perspectives

Further large cohort studies are needed to assess the association between (T>A, -76) mutation and *H. pylori* infection (susceptibility and progression). Also, further studies are encouraged to investigate the novel mutation (A>T, +27) in terms of the frequency of the minor allele (T) in the Sudanese population and its functional significance using computational and experimental approaches. Identifying which of these detected variants are functional is of great relevance for discovering new preventive, diagnostic and therapeutic strategies against the incidence and/or progression of multifactorial diseases such as *H. pylori* infection.

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ORIGINAL ARTICLE

Prospective Study

Outreach onsite treatment with a simplified pangenotypic direct-acting anti-viral regimen for hepatitis C virus micro-elimination in a prison

Chun-Ting Chen, Ming-Ying Lu, Meng-Hsuan Hsieh, Pei-Chien Tsai, Tsai-Yuan Hsieh, Ming-Lun Yeh, Ching-I Huang, Yi-Shan Tsai, Yu-Min Ko, Ching-Chih Lin, Kuan-Yu Chen, Yu-Ju Wei, Po-Yao Hsu, Cheng-Ting Hsu, Tyng-Yuan Jang, Ta-Wei Liu, Po-Cheng Liang, Ming-Yen Hsieh, Zu-Yau Lin, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Yu-Lueng Shih, Ming-Lung Yu

ORCID number: Chun-Ting Chen 0000-0002-6218-2300; Ming-Ying Lu 0000-0002-1317-9586; Meng-Hsuan Hsieh 0000-0001-9739-3901; Pei-Chien Tsai 0000-0002-5044-6727; Tsai-Yuan Hsieh 0000-0003-4211-7015; Ming-Lun Yeh 0000-0003-3728-7618; Ching-I Huang 0000-0002-5467-0398; Yi-Shan Tsai 0000-0002-5251-1318; Yu-Min Ko 0000-0002-8424-9289; Ching-Chih Lin 0000-0002-4829-308X; Kuan-Yu Chen 0000-0001-9350-2699; Yu-Ju Wei 0000-0003-1266-7796; Po-Yao Hsu 0000-0002-5443-7203; Cheng-Ting Hsu 0000-0002-9057-3536; Tyng-Yuan Jang 0000-0003-2961-130X; Ta-Wei Liu 0000-0002-6978-9922; Po-Cheng Liang 0000-0001-9189-6604; Ming-Yen Hsieh 0000-0002-8019-3011; Zu-Yau Lin 0000-0002-8489-7147; Chung-Feng Huang 0000-0002-3367-068X; Jee-Fu Huang 0000-0002-2752-7051; Chia-Yen Dai 0000-0003-2296-3054; Wan-Long Chuang 0000-0002-2376-421X; Yu-Lueng Shih 0000-0003-4629-4393; Ming-Lung Yu 0000-0001-8145-1900.

Author contributions: Chen CT and Yu ML drafted the manuscript; Tsai PC and Hsieh MH assisted with data collection and analysis; Yu ML and Shih YL made equal

Chun-Ting Chen, Yu-Lueng Shih, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tri-Service General Hospital Penghu Branch, National Defense Medical Center, Penghu County 88041, Taiwan

Chun-Ting Chen, Tsai-Yuan Hsieh, Yu-Lueng Shih, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei 11490, Taiwan

Ming-Ying Lu, Meng-Hsuan Hsieh, Pei-Chien Tsai, Ming-Lun Yeh, Ching-I Huang, Yi-Shan Tsai, Yu-Min Ko, Ching-Chih Lin, Kuan-Yu Chen, Yu-Ju Wei, Po-Yao Hsu, Cheng-Ting Hsu, Tyng-Yuan Jang, Ta-Wei Liu, Po-Cheng Liang, Ming-Yen Hsieh, Zu-Yau Lin, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Ming-Lung Yu, Division of Hepatobiliary, Department of Internal Medicine and Hepatitis Center, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

Meng-Hsuan Hsieh, Ming-Lun Yeh, Ching-I Huang, Yi-Shan Tsai, Yu-Min Ko, Ching-Chih Lin, Kuan-Yu Chen, Yu-Ju Wei, Po-Yao Hsu, Cheng-Ting Hsu, Tyng-Yuan Jang, Ta-Wei Liu, Po-Cheng Liang, Ming-Yen Hsieh, Zu-Yau Lin, Chung-Feng Huang, Jee-Fu Huang, Chia-Yen Dai, Wan-Long Chuang, Ming-Lung Yu, School of Medicine and Hepatitis Research Center, College of Medicine, and Center for Liquid Biopsy and Cohort Research, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

Ming-Lung Yu, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

Corresponding author: Ming-Lung Yu, MD, PhD, Adjunct Professor, Chief Doctor, Full Professor, Division of Hepatobiliary, Department of Internal Medicine and Hepatitis Center, Kaohsiung Medical University Hospital, No. 100 Shin-Chuan 1st Road, Sanmin District, Kaohsiung 80708, Taiwan. fish6069@gmail.com

Abstract

BACKGROUND

Prisoners are at risk of hepatitis C virus (HCV) infection, especially among the

contributions; all authors participated in universal mass screening, immediate onsite treatment, read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (IRB: KMUHIRB-SV(I)-20190033) and the Institutional Review Board of Tri-Service General Hospital (IRB: TSGHIRB 2-107-05-080).

Conflict-of-interest statement: No author had reported a potential conflict of interest relevant to this work.

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people who inject drugs (PWID). We implemented an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pan-genotypic direct-acting antivirals (DAA) regimen, 12 wk of sofosbuvir/velpatasvir, in a PWID-dominant prison in Taiwan.

AIM
To implement an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pan-genotypic DAA regimen in a PWID-dominant prison in Taiwan.

METHODS
HCV-viremic patients were recruited for onsite treatment program for HCV micro-elimination with a pangenotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, from two cohorts in Penghu Prison, either identified by mass screen or in outpatient clinics, in September 2019. Another group of HCV-viremic patients identified sporadically in outpatient clinics before mass screening were enrolled as a control group. The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV ribonucleic acid (RNA) 12 wk after end-of-treatment).

RESULTS
A total of 212 HCV-viremic subjects were recruited for HCV micro-elimination campaign; 91 patients treated with sofosbuvir/Ledipasvir or glecaprevir/pibrentasvir before mass screening were enrolled as a control. The HCV micro-elimination group had significantly lower proportion of diabetes, hypertension, hyperlipidemia, advanced fibrosis and chronic kidney diseases, but higher levels of HCV RNA. The SVR12 rate was comparable between the HCV micro-elimination and control groups, 95.8% (203/212) *vs* 94.5% (86/91), respectively, in intent-to-treat analysis, and 100% (203/203) *vs* 98.9% (86/87), respectively, in per-protocol analysis. There was no virological failure, treatment discontinuation, and serious adverse event among sofosbuvir/velpatasvir-treated patients in the HCV micro-elimination group.

CONCLUSION
Outreach mass screening followed by immediate onsite treatment with a simplified pangenotypic DAA regimen, sofosbuvir/velpatasvir, provides successful strategies toward HCV micro-elimination among prisoners.

Key Words: Direct-acting antivirals; Sofosbuvir; Velpatasvir; People who inject drugs; Universal screen

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Core Tip: We implemented an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pangenotypic direct-acting antivirals egimen, 12 wk of sofosbuvir/velpatasvir, in a people who inject drugs (PWID)-dominant prison. Our study achieved high sustained virological response rate in HCV-infected PWID-dominant prisoners. We provided successful strategies toward HCV micro-elimination among prisoners.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a progressive and blood-borne infectious disease that can lead to end stage liver diseases, such as hepatic decompensation, liver cirrhosis, and hepatocellular carcinoma[1,2]. Iatrogenic transmission of HCV, such as blood transfusion and surgery, has decreased in developed countries. Whereas people who inject drugs (PWID) has become the major population of HCV transmission, which could consist of approximately 80% of HCV-infected patients[3]. Given that lack of vaccine available, "treatment as prevention" for HCV transmission in PWID is very important for HCV elimination.

Prisoners are at high risk of HCV infection, with prevalence rates ranging from 3.1% to 38%[4,5]. The high prevalence of HCV infection in prisoners is resulted from unsafe lifestyles, psychiatric disorders, and social problems before they are incarcerated. Recently, PWID has been the most important risk factor of HCV infection in prisoners [6]. The anti-HCV prevalence rate could be as high as 91% among PWID prisoners[7]. Screening and eliminating HCV infection in prisoners is therefore an important social health issue.

According to the American Association for the Study of Liver Diseases and European Association for the Study of the Liver (EASL) guidelines, all HCV viremic patients should be treated if life span is expected more than one year[8,9]. HCV therapeutic strategies have been revolutionized significantly because of the availability of direct-acting antivirals (DAA)[10]. Interferon (IFN)-based regimens for HCV infection have serious side effects, long therapeutic duration, and contraindications, leading to the huge gaps in HCV care cascade[11]. The current IFN-free DAA regimens provide shorter treatment duration, very high treatment efficacy and safety profiles, not only for general population[12], but also for special populations[13], such as HCV/human immunodeficiency virus (HIV) coinfected patients, hepatitis B virus (HBV)/HCV coinfected patients and patients with chronic kidney diseases in real-world clinical settings[14,15].

World Health Organization (WHO) set a global goal of HCV elimination by 2030 [16], and Taiwan authority is even ambitious by 2025[17]. To achieve the goal, implementation of the concept of HCV micro-elimination is regarding as an efficient and practical strategy[18]. We have proved that "universal mass screening plus outreach onsite treatment" is the key to achieve HCV micro-elimination among patients under maintenance hemodialysis[19].

Recently, the latest EASL HCV guideline recommended simplified, genotyping/subtyping-free, pangenotypic anti-HCV treatment, either sofosbuvir/ velpatasvir or glecaprevir/pibrentasvir, to increase the accessibility and global cure rates among patients with > 12 years, chronic hepatitis C without cirrhosis or with compensated cirrhosis, with or without HIV co-infection, whatever treatment-naïve or IFN-experienced[8].

Since HCV treatment is not frequently administered to prisoners due to unawareness of HCV infection, difficultly management, easily loss to follow-up, and lack of hepatologist in prison[20], collaboration between hepatologists and prison authorities to carry out strategies for HCV diagnosis and treatment in prisoners in highly demanded. Herein, we implemented an outreach strategy in combination with universal mass screen and onsite treatment with a simplified pan-genotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, toward HCV micro-elimination in a PWID-dominant prison in Taiwan.

MATERIALS AND METHODS

Patients linked to onsite treatment program for HCV micro-elimination

HCV-viremic patients were recruited from two cohorts in Penghu Prison (Agency of Corrections, Ministry of Justice, Taiwan), a PWID-dominant prison (Figure 1).

HCV-viremic patients identified by a universal mass screening

In September 2019, we conducted a 5 d universal mass screening of viral hepatitis in Penghu Prison. These inclusion criteria were prisoners, who were at least 20 years old, being willing to enter the study for screening of viral hepatitis. The study of mass screening was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (IRB: KMUHIRB-SV(I)-20190033). All participants provided written informed consents. A total of 1137 subjects from 1697 inmates participated the mass screening[21]. Among them, 396 (34.8%) subjects had anti-HCV seropositivity;

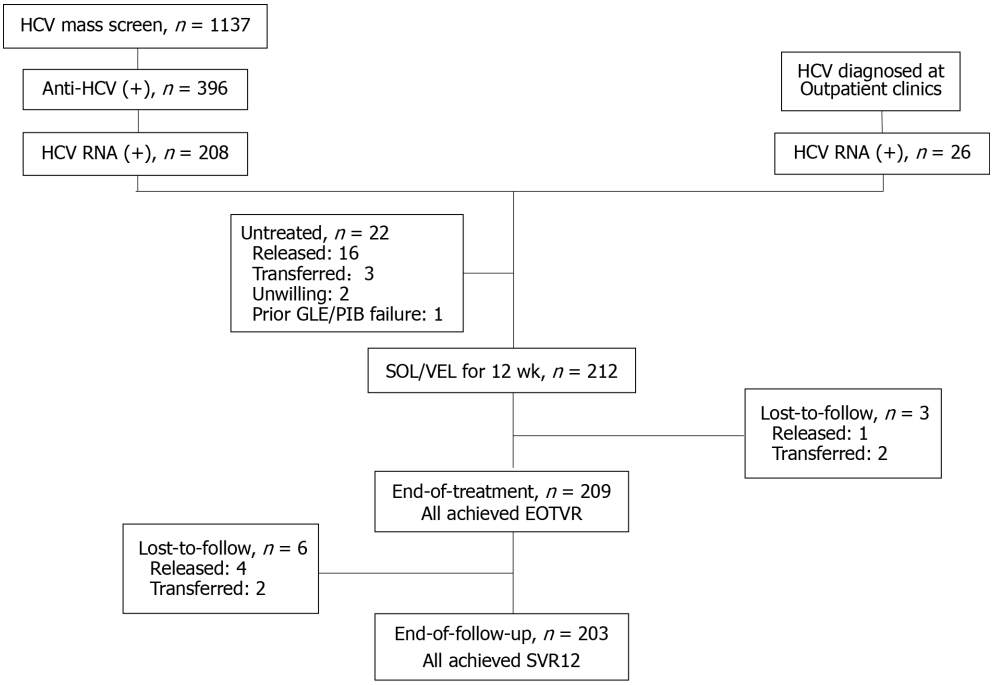


Figure 1 Patient flowchart of hepatitis C virus treatment with a simplified pan-genotypic directly-acting antivirals regimen in Penghu Prison. HCV: Hepatitis C virus; DAA: Directly-acting antivirals; SOL/VEL: Sofosbuvir/velpatasvir; GEL/PIB: Glecaprevir/pibrentasvir; EOTVR: Virological response at end-of-treatment; SVR12: Sustained viral response at post-treatment wk 12.

208 (52.5%) of the 396 subjects were seropositive for HCV ribonucleic acid (RNA) and linked to the onsite HCV treatment program with universal sofosbuvir/velpatasvir regimen.

HCV-viremic patients identified in outpatient clinics during the period of HCV mass screening

Another 26 HCV-viremic subjects identified in outpatient clinics of Penghu Prison between August to December 2019 were also linked to the onsite HCV treatment program with universal sofosbuvir/velpatasvir regimen.

All patients received pretreatment evaluation in December 2019, including medical history, liver and renal function tests, complete blood cell counts, HCV viral loads and genotyping, abdominal sonography and assessment of potential drug–drug interactions. A 12 wk, oral pan-genotypic regimen of sofosbuvir/velpatasvir 400/100 mg fixed-dose combination once daily was initiated in January-February 2020.

Patients identified and treated by DAAs in outpatient clinics before mass screening

A total of 91 HCV-viremic patients identified in outpatient clinics of Penghu Prison and treated with DAA before mass screening from 2017 to 2019 were enrolled as a control. The selection of DAA regimens were based on physician's discretion according to the viral genotype and criteria of reimbursement of National Health Insurance Administration, Taiwan. All patients received pretreatment evaluation, including medical history, liver and renal function tests, complete blood cell counts, HCV viral loads and genotyping, abdominal sonography and assessment of potential drug–drug interactions.

All participants signed informed consent forms. These enrolled inmates of our study were protected according to the guidelines of the Declaration of Helsinki. The current study of DAA therapy was approved by the Institutional Review Board of Tri-Service General Hospital (IRB: TSGHIRB 2-107-05-080).

Assessment, monitoring and endpoints

Anti-HCV antibody was determined by the third generation, commercially available immunoassay (Ax SYM HCV III; Abbott Laboratories, North Chicago, IL). HCV RNA viral loads and genotype were determined by real-time PCR assays [RealTime HCV; Abbott Molecular, Des Plaines IL, United States; detection limit: 12 IU/mL])[22]. Liver cirrhosis was defined by the presence of clinical, radiological, endoscopic or laboratory evidence of cirrhosis and/or portal hypertension or fibrosis-4 index (FIB-4) (> 6.5).

Laboratory data monitoring and assessment of side effects were performed at treatment wk 2, 4, 8 and end-of-treatment (EOT), and 12 wk after EOT.

The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV RNA throughout 12 wk of the post-treatment follow-up period).

Statistical analyses.

The efficacy of all DAA regimens was determined in a intent-to-treat (ITT) population (all enrolled patients with at least one dose of DAA) and a per-protocol (PP) population (subjects receiving at least one dose of DAA and retained in Penghu Prison throughout the DAA treatment and follow-up period). Safety assessments reported adverse event (AE), serious adverse event (SAE) and laboratory abnormalities in the ITT population. Continuous variables are expressed as means ± standard deviation (SD), and categorical variables are expressed as percentages. The differences of continuous variables are estimated by the Student's *t* test. The differences in categorical variables are analyzed using the Chi-square test. The on-treatment and off-treatment virological response rates were analyzed in number and percentages with 95% confidence interval (CI). All data analyses were performed using the SPSS software version 18.0 (SPSS Inc., Chicago, Illinois, United States).

RESULTS

Patient flowchart of HCV micro-elimination campaign

The patient flowchart of HCV mass screen, assessment and treatment was shown in Figure 1. A total of 234 HCV-viremic patients, 208 from mass screening and 26 from outpatient clinics in Penghu Prison were assessed for eligibility of group therapy with sofosbuvir/velpatasvir in December 2019. Twenty-two patients were excluded from anti-HCV therapy due to scheduled to be released from jail (n = 16) or transferred to other jails (n = 3) within 6 mo, unwilling to receive therapy (n = 2) and prior glecaprevir/pibrentasvir treatment failure (n = 1). Finally, 212 patients were recruited for sofosbuvir/velpatasvir therapy initiated in January-February 2020.

Patient characteristics

The baseline characteristics of 303 HCV-viremic patients, including 212 in HCV micro-elimination campaign and 91 sporadic controls from outpatient clinics before micro-elimination campaign were listed in Table 1. They mean age was 48.4 years with male dominant (99.7%). Thirty (9.9%) had HBV coinfection. The mean FIB-4 was 1.3, with 20 (6.6%) had advanced fibrosis (FIB-4 > 3.25). Only one patient (0.3%) had liver cirrhosis. The mean HCV RNA levels was 6.5 Logs IU/mL, dominant with HCV genotype 1 (HCV-GT1, 42.2%), followed by HCV-GT6 (35.3%), HCV-GT3 (11.6%) and HCV-GT2 (10.6%). Three (1%) patients were prior IFN-experienced. The two groups had comparable characteristics in terms of age, gender, HBV co-infection, liver and renal function tests, FIB-4 score, HCV genotype distribution, and prior history of IFN-based therapy. However, the sporadic patients identified in outpatient clinics had significantly higher proportion of comorbidities, including diabetes, hypertension, hyperlipidemia and an estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, but significantly lower HCV viral loads. None of patient had decompensated cirrhosis nor liver cancer.

Treatment efficacy

All of 212 patients in HCV micro-elimination campaign received sofosbuvir/velpatasvir treatment; while among 91 sporadic patients with DAA therapy before HCV micro-elimination campaign, 78 (85.7%) received 12 wk of sofosbuvir/Ledipasvir and 13 (14.3%) received 8-12 wk of glecaprevir/pibrentasvir according to the Taiwan HCV guideline[12,13].

In ITT analysis, the overall SVR12 rate was 95.4% (289/303) with comparable SVR12 rates between sporadic HCV control group (94.5%, 86/91) and HCV micro-elimination group (95.8%, 203/212, P = 0.126, Table 2).

During DAA treatment period, all of patients in sporadic HCV control group completed DAA therapy, while 3 patients in HCV micro-elimination group lost-to-follow (2 transferred; 1 released). During the post-treatment follow-up period, 4 patients in sporadic HCV control group lost-to-follow (4 released), while 6 patients in HCV micro-elimination group lost-to-follow (2 transferred; 4 released). In PP analysis, the overall SVR12 rate was 99.7% (289/290) with comparable SVR12 rates between

Table 1 Baseline characteristics of hepatitis C virus-infected patients receiving directly-acting antivirals therapy between sporadic hepatitis C virus therapy in outpatient clinics and campaign of hepatitis C virus micro-elimination in Penghu prison

	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination (January 1, 2020 - March 31, 2020)	*P* value
n	303	91	212	-
Age (yr)	48.4 ± 8.2	47.6 ± 8.7	48.7 ± 8.0	0.271
Male	303 (99.7)	90 (98.9)	212 (100.0)	0.126
[1]BMI, kg/m^2	23.9 ± 3.2	23.9 ± 3.3	23.9 ± 3.2	0.986
> 27 kg/m^2	34 (13.8)	11 (13.9)	23 (13.4)	0.960
Diabetes	10 (3.3)	8 (8.8)	2 (0.9)	0.0005[a]
Hypertension	59 (19.5)	25 (27.5)	34 (16.0)	0.021[a]
Hyperlipidemia	8 (2.6)	7 (7.7)	1 (0.5)	0.0003[a]
Cardiovascular disease	2 (0.7)	1 (1.1)	1 (0.5)	0.537
HBsAg (+)	30 (9.9)	9 (9.9)	21 (9.9)	0.997
AST, IU/L	41.3 ± 35.5	45.9 ± 38.9	39.4 ± 33.8	0.168
ALT, IU/L	65.4 ± 77.4	71.6 ± 69.8	62.7 ± 80.4	0.329
Abnormal AST or ALT	159 (52.5)	54 (59.3)	105 (49.5)	0.117
White cell count, × 10^3/iL	6.6 ± 1.9	6.4 ± 2.0	6.7 ± 1.8	0.188
Hemoglobin concentration, g/dL	15.9 ± 1.3	16.0 ± 1.3	15.9 ± 1.3	0.762
Platelet count, × 10^{3}u/L	227.6 ± 67.4	219.4 ± 72.1	231.2 ± 65.1	0.181
Albumin, g/dl	4.5 ± 0.3	4.5 ± 0.4	4.5 ± 0.2	0.233
Total bilirubin, mg/dL	0.8 ± 0.3	0.9 ± 0.4	0.8 ± 0.3	0.003[a]
LC	1 (0.3)	1 (1.1)	0 (0.0)	0.300
FIB-4	1.3 ± 1.0	1.5 ± 1.4	1.2 ± 0.8	0.096
> 3.25	20 (6.6)	10 (11.0)	10 (4.7)	0.044[a]
eGFR, mL/min/1.73 m^2	99.9 ± 17.7	99.1 ± 21.0	100.3 ± 16.4	0.624
< 60	4 (1.3)	3 (3.3)	1 (0.4)	0.048[a]
HCV RNA, $\log_{10}$ IU/mL	6.5 ± 1.1	6.0 ± 1.0	6.7 ± 1.1	< 0.001[a]
HCV genotype, 1/2/1+2/3/6	128 (42.2)/32 (10.6)/1 (0.3)/35 (11.6)/107 (35.3)	38 (41.8)/9 (9.9)/0/11 (12.1)/33 (36.2)	90 (42.5)/23 (10.8)/1 (0.5)/24 (11.3)/74 (34.9)	0.968
DAA regimen				
SOF/VEL	212 (70.0)	0 (0.0)	212 (100.0)	< 0.001[a]
SOF/LDV	78 (25.7)	78 (85.7)	0 (0.0)	
GLE/PIB	13 (4.3)	13 (14.3)	0 (0.0)	
Prior treatment history				
Naïve	300 (99.0)	89 (97.8)	211 (99.5)	0.216
Experienced-IFN	3 (1.0)	2 (2.2)	1 (0.5)	

[1]56 patients did not have body mass index information (12 patients before campaign of hepatitis C virus (HCV) micro-elimination; 44 patients in campaign of HCV micro-elimination).

[a]*P* < 0.05. DAA: Directly-acting antivirals; HCV: Hepatitis C virus; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LC: Liver cirrhosis; FIB-4: Fibrosis-4 index; HBsAg: Hepatitis B surface antigen; eGFR: Estimated glomerular filtration rate (mL/min/1.73 m^2); SOF: Sofosbuvir; VEL: Velpatasvir; LDV: Ledipasvir; GLE: Glecaprevir; PIB: Pibrentasvir; IFN: Interferon.

Table 2 Virological responses of hepatitis C virus-infected patients receiving directly-acting antivirals therapy before and during campaign of hepatitis C virus micro-elimination in Penghu prison in Penghu prison

Undetectable HCV RNA, *n*/N (%)	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination with simplified pan-genotypic SOF/VEL regimen (January 1, 2020 - March 31, 2020)	*P* value
Intention-to-treat population				
Treatment 4 wk	284/303 (93.7)	85/91 (93.4)	199/212 (93.9)	0.879
End-of-treatment	300/303 (99.0)	91/91 (100.0)	209/212 (98.6)	0.557
End-of 12 wk follow-up	289/303 (95.4)	86/91 (94.5)	203/212 (95.8)	0.126
Per-protocol population				
Treatment 4 wk	284/301 (94.4)	85/90[1] (94.4)	199/211[2] (94.3)	0.964
End-of-treatment	300/300 (100.0)	91/91 (100.0)	209/209[3] (100.0)	-
End-of 12 wk follow-up	289/290 (99.7)	86/87[4] (98.9)e[5]	203/203[6] (100.0)	0.126

[1]One missing data.
[2]One transferred; One missing data.
[3]Two transferred; One released.
[4]Four released.
[5]One relapser.
[6]Four transferred; Five released. HCV: Hepatitis C virus; VEL: Velpatasvir; SOF: Sofosbuvir.

sporadic HCV control group (98.9%, 86/87) and HCV micro-elimination group (100%, 203/203, $P = 0.126$, Table 2). Only one patient experienced virological failure (54 years old male, treatment-naïve, HCV-GT3 infection with baseline viral loads of 62,883 IU/mL and FIB-4 of 2.37; relapsed from a 12 wk regimen of glecaprevir/pibrentasvir).

Safety profiles

The safety profiles of both groups were shown in Table 3. None of patients had treatment discontinuation other than released or transferred. None experienced serious adverse event. The frequency of adverse events was 4.3% (4/91) and 1.4% (3/212), respectively, among patients in sporadic control group and HCV micro-elimination group. The most reported adverse events were rash in 3 of 13 (23.1%) patients treated with glecaprevir/pibrentasvir and pruritus in 2 of 212 (0.9%) patients treated with sofosbuvir/velpatasvir. None of patients experienced grade 3 or 4 Laboratory abnormality.

DISCUSSION

In the current study, we demonstrated that mass screening combined with onsite group therapy by using a simplified pan-genotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, provides an "one-size fits all" solution toward the achievement of HCV micro-elimination in prisoners. The SVR rate was 95.6% in ITT population and 100% in PP population after excluding the inmates released or transferred before end-of-follow-up. The high SVR rate was observed in this PWID-dominant population, which HCV genotype distribution was diverse, including genotypes 1a, 1b, 2, 3 and 6.

Recent advance in the development of IFN-free pan-genotypic DAA regimens has remarkably improved the treatment efficacy with an overall SVR rates of > 90%. Therefore, WHO set the global of HCV elimination by 2030, through the achievement of > 90% diagnosis rate and > 80% treatment rate for eligible patients[16]. Nevertheless, there are many barriers in each HCV care cascade toward HCV

Table 3 Safety profiles of hepatitis C virus-infected patients receiving direct-acting antivirals therapy in Penghu prison

n (%)	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination with simplified pan-genotypic SOF/VEL regimen (January 1, 2020 - March 31, 2020)
n	303	91	212
Treatment discontinuation other than released or transferred	0 (0.0)	0 (0.0)	0 (0.0)
Serious adverse events	0 (0.0)	0 (0.0)	0 (0.0)
Death	0 (0.0)	0 (0.0)	0 (0.0)
Adverse events	7 (2.3)	4 (4.3)	3 (1.4)
Fatigue	0 (0.0)	0 (0.0)	0 (0.0)
Pruritus	2 (0.7)	0 (0.0)	2 (0.9)
Rash	3 (1.0)	3 (3.2)	0 (0.0)
Nausea	0 (0.0)	0 (0.0)	0 (0.0)
Anorexia	0 (0.0)	0 (0.0)	0 (0.0)
Constipation	0 (0.0)	0 (0.0)	0 (0.0)
Dizziness	0 (0.0)	0 (0.0)	0 (0.0)
Insomnia	0 (0.0)	0 (0.0)	0 (0.0)
Headache	1 (0.3)	0 (0.0)	1 (0.5)
Others	1 (0.3)	1 (1.0)	0 (0.0)
Grade 3 or 4 laboratory abnormalities			
Total blood bilirubin	0 (0.0)	0 (0.0)	0 (0.0)
Alanine aminotransferase	0 (0.0)	0 (0.0)	0 (0.0)

DAA: Directly-acting antivirals; HCV: Hepatitis C virus; VEL: Velpatasvir; SOF: Sofosbuvir.

elimination at the population level[11,23]. To overcome the barriers, combining the concept of micro-elimination and an outreach strategy with immediate onsite treatment would be a more efficient and practical approach to achieve that goal[18,24]. The current study compared the HCV-infected inmates identified sporadically in outpatient clinics of Penghu Prison from 2017 to 2019 before mass screening and the patients identified by mass screening. We found that mass screening identified 208 HCV-viremic patients in a 5 d screening program from 1137 inmates (encountered around two-third of total inmates in Penghu Prison), compared to 91 HCV-viremic inmates treated in outpatient clinics from 2017 to September 2019. Our results demonstrated that mass screening with immediate onsite treatment provide much more efficient and practical solution to overcome the gaps of disease awareness and link-to-care in the HCV care cascades toward HCV micro-elimination in prisoners. In addition, we implemented "HCV reflex testing" in the mass screening program to scale-up and speed-up the diagnosis and link-to-care for treatment uptake of HCV infections[25].

PWID is known as the major risk factor of HCV infection and transmission. Although the anti-HCV prevalence in PWID prisoners decreased from 91% in 2014 to 34.8% in 2019 by the strategy of safe injection in Taiwan[21], almost all (97.6%) of HCV-infected prisoners were PWID. Given the lack of vaccine available and high risk of transmission, the strategy of universal screening and concept of "treatment as prevention" are the keys to HCV elimination in prison as well as PWID.

We observed that the sporadic HCV-infected prisoners identified in outpatient clinics had significantly higher proportion of comorbidities, including diabetes, hypertension, hyperlipidemia and eGFR, than those participating in the HCV micro-elimination campaign. It implicated that a great proportion of identified sporadically in outpatient clinics were due to concomitant morbidities; by contrast, many HCV-infected patients were unaware to their HCV diseases. In our mass screening, only 36.6% (145/396) of HCV-infected prisoners were aware of HCV infection before screening[21]. It indicates that the implementation of an outreach strategy with universal mass screen is necessary for HCV micro-elimination in prison.

Despite of the advances in the management of HCV infections, DAA therapy in incarcerated HCV-infected people remains many obstacles to be resolved, including disease unawareness, lack of updated information about the benefits of new DAA treatment, uncertainty of treatment right[26], poor accessibility due to of onsite treatment facilities or HCV treaters. Another difficulty for HCV treatment in prisoners is the unexpected or scheduled releasing from prison or transferring to other prisons, which frequently leads to the interruption of treatment or lost-to- follow up[20,27]. We are lucky that the Taiwan Health Insurance covered all incarcerated people, including all of the laboratory tests and ultrasound sonography and the cost of DAA regimens. Each prison has a contracted hospital providing point-of-care facility. Before initiating DAA therapy, we excluded the patients with expected release or transfer within 24 wk, and negotiated with the authority to avoid unnecessary transferring to other prisons during the period of HCV treatment and follow-up once the inmates entering the DAA course. Eventually we achieved a high treatment rate of 90.6% (212/234) and a high treatment complete rate of 95.8% (203/212), with a high cure rate at 100% (212/212).

Before the IFN-free DAA available, the lower SVR rate, much longer treatment duration and frequent adverse events of IFN-based treatment discouraged HCV-infected prisoners from receiving treatment[10]. IFN-free DAA regimens revolutionized HCV treatment which has largely extended the indication for various HCV-infected patients. Nevertheless, the application of typical DAA regimens are based on HCV genotype, presence of decompensated cirrhosis, renal function, and prior treatment experience. The two pangenotypic DAA regimens, sofosbuvir/velpatasvir, and glecaprevir/pibrentasvir, have achieved very high SVR rates of > 95%, regardless of HCV GTs, except for treatment-experienced cirrhotic HCV GT3 patients or GT3b patients[8,12,13]. Recently, to improve the access to anti-HCV therapy, reduce the cost of laboratory tests and the relative complexity of genotype-based treatment strategies, simplified treatment without many information needed for treatment decision are recommended to facilitate the care cascade among populations who are historically less engaged in healthcare, such as PWIDs and prisoners[8]. EASL recommends simplified, genotyping/subtyping-free regimens for IFN-free DAA treatment-naïve (except sofosbuvir plus ribavirin), HCV-infected or HCV-HIV coinfected adolescent and adult patients without cirrhosis or with compensated cirrhosis, regardless of HCV genotypes[8]. These recommendations are a universal 12 wk regimen of sofosbuvir/velpatasvir for all patients or glecaprevir/pibrentasvir, 8 wk for non-cirrhotic, 12 wk for compensated cirrhotic, and 16 wk for HCV GT3 patients, respectively. There are only four information needed before treatment, including the presence of HCV viremia, potential drug-drug interactions, and prior treatment experience, and presence of cirrhosis. The advantages of glecaprevir/pibrentasvir is a shorter 8 wk regimen for treatment-naïve HCV patients and IFN-experienced non-cirrhotic patients with compensated liver diseases, which would be benefit for prisoners who are expected to be released or transferred in a short term. However, glecaprevir, a protease inhibitor, is contraindicated for patients with hepatic decompensation and at risk for rare occurrence of serious drug-induced liver injury[28]. Also, glecaprevir/pibrentasvir has higher pill burden, three tablets a d. The advantages of sofosbuvir/velpatasvir include a universal fixed 12 wk regimen, one tablet a d, for all HCV patients with compensated liver diseases, less frequency of potential drug-drug interactions[29], and safety for those with hepatic decompensation. However, a 12 wk regimen with sofosbuvir/velpatasvir needs one more visit and monitoring when compared to an 8 wk regimen with glecaprevir/pibrentasvir. Therefore, we select sofosbuvir/velpatasvir as the antiviral regimen for our outreach onsite treatment. In

our study, all HCV-viremic prisoners fit the criteria of simplified, genotyping/subtyping-free regimens, except one who failed to prior glecaprevir/pibrentasvir therapy and was not enrolled for sofosbuvir/velpatasvir treatment. In our PP analysis, the overall SVR12 rate was comparable between HCV patient group (98.9%, 86/87) and HCV micro-elimination group (100%, 203/203). Our study provided evidence for the concept that simplified, genotyping/subtyping-free regimens can achieve high SVR12 rate in HCV-infected PWID-dominant prisoners.

In our study, none of prisoners had DAA treatment discontinuation due to adverse events. None experienced serious adverse event. These data indicated that the simplified, genotyping/subtyping-free regimen, sofosbuvir/velpatasvir, was safe and well tolerated for HCV-infected PWID-dominant prisoners. Very few adverse events were reported in both groups, whatever using sofosbuvir/Ledipasvir, glecaprevir/pibrentasvir and sofosbuvir/velpatasvir, when compared to the data from clinical trials[30,31]. It might be due to that current population was younger and less patients with advanced fibrosis or chronic kidney diseases.

There were some limitations in our study. First, not all inmates in Penghu Prison participated our mass screening. Strategies and policy to encourage inmates to receive HCV screening is mandatory to achieve the goal of WHO. Second, unexpected prisoners' transferral and release could not be completely avoided, which caused incomplete treatment and follow-up. Successfully linking the released or transferred people to another HCV treaters could help completing HCV treatment and follow-up. Third, there was no reimbursement for the retreatment of prior DAA failed patients in Taiwan at the time of the current study.

CONCLUSION

Well-designed strategies for mass screening and treatment for HCV-infected prisoners can be implemented successfully by the collaboration between physicians and prison authorities. We demonstrated that mass screening followed by immediate onsite treatment with a simplified pangenotypic DAA regimen, sofosbuvir/velpatasvir, provides successful strategies toward HCV micro-elimination among prisoners.

ARTICLE HIGHLIGHTS

Research background

Prisoners are at high risk of hepatitis C virus (HCV) infection. To screen and treat HCV infection in prisoners is an important social health issue. It can be the start for HCV micro-elimination.

Research motivation

HCV treatment is not frequently administered to prisoners due to multiple factors. Therefore, we implemented an outreach strategy in combination with universal mass screen and onsite treatment in a prison.

Research objectives

To implement an outreach strategy. HCV-infected prisoners received a simplified pan-genotypic direct-acting antivirals (DAA) regimen, 12 wk of sofosbuvir/velpatasvir. The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV RNA throughout 12 wk of the post-treatment follow-up period).

Research methods

All participants received blood tests. We used reflex testing. All HCV-infected prisoners received DAA therapy. Laboratory data monitoring and assessment of side effects were performed at treatment wk 2, 4, 8 and end-of-treatment (EOT), and 12 wk after EOT.

Research results

DAA regimen with sofosbuvir/velpatasvir achieved high SVR12 rate. There was no virological failure, treatment discontinuation, and serious adverse event among sofosbuvir/velpatasvir-treated patients in the HCV micro-elimination group.

Research conclusions

Well-designed strategies for mass screening and treatment for HCV-infected prisoners can be implemented successfully by the collaboration between physicians and prison authorities.

Research perspectives

Our study provided evidence for the concept that simplified, genotyping/subtyping-free regimens can achieve high SVR12 rate in HCV-infected prisoners. In the future, it is possible to implement the strategy to all prisoners in our country.

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Contents

Weekly Volume 28 Number 3 January 21, 2022

FRONTIER

275 Endothelial cells and blood vessels are major targets for COVID-19-induced tissue injury and spreading to various organs

Tarnawski AS, Ahluwalia A

290 Eliminating viral hepatitis in children after liver transplants: How to reach the goal by 2030

Sintusek P, Thanapirom K, Komolmit P, Poovorawan Y

REVIEW

310 Non-alcoholic fatty liver disease and hepatocellular carcinoma: Clinical challenges of an intriguing link

Chrysavgis L, Giannakodimos I, Diamantopoulou P, Cholongitas E

MINIREVIEWS

332 Endoscopic ultrasound role in pancreatic adenocarcinoma treatment: A review focusing on technical success, safety and efficacy

Sbeit W, Napoléon B, Khoury T

ORIGINAL ARTICLE

Basic Study

348 Melatonin prevents oxidative stress, inflammatory activity, and DNA damage in cirrhotic rats

Colares JR, Hartmann RM, Schemitt EG, Fonseca SRB, Brasil MS, Picada JN, Dias AS, Bueno AF, Marroni CA, Marroni NP

Case Control Study

365 Microbiome changes in the gastric mucosa and gastric juice in different histological stages of *Helicobacter pylori*-negative gastric cancers

Sun QH, Zhang J, Shi YY, Zhang J, Fu WW, Ding SG

Retrospective Cohort Study

381 Postoperative mortality and morbidity after D2 lymphadenectomy for gastric cancer: A retrospective cohort study

Brisinda G, Chiarello MM, Crocco A, Adams NJ, Fransvea P, Vanella S

LETTER TO THE EDITOR

399 Prophylactic drains in totally laparoscopic distal gastrectomy: are they always necessary?

Manzia TM, Parente A, Angelico R

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Weekly Volume 28 Number 3 January 21, 2022

Contents

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FRONTIER

Endothelial cells and blood vessels are major targets for COVID-19-induced tissue injury and spreading to various organs

Andrzej S Tarnawski, Amrita Ahluwalia

ORCID number: Andrzej S Tarnawski 0000-0002-6813-6698; Amrita Ahluwalia 0000-0002-2064-5725.

Author contributions: Ahluwalia A and Tarnawski AS designed the overall concept, outline of the manuscript, and contributed to the writing and editing of the manuscript, illustrations, and review of literature, and approved the final version of manuscript. Ahluwalia A (amrita.ahluwalia@va.gov) and Tarnawski AS (atarnawski@yahoo.com) are co-corresponding authors.

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Andrzej S Tarnawski, Gastroenterology Research Department, University of California Irvine and the Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States

Amrita Ahluwalia, Research Service, Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States

Corresponding author: Amrita Ahluwalia, PhD, Research Scientist, Research Service, Veterans Administration Long Beach Healthcare System, 5901 E, Seventh Street, Long Beach, CA 90822, United States. amrita.ahluwalia@va.gov

Abstract

The coronavirus disease 2019 (COVID-19) infected so far over 250 million people and caused the death of over 5 million worldwide. Aging, diabetes, and cardiovascular diseases, conditions with preexisting impaired endothelial functions predispose to COVID-19. While respiratory epithelium is the main route of virus entry, the endothelial cells (ECs) lining pulmonary blood vessels are also an integral part of lung injury in COVID-19 patients. COVID-19 not only affects the lungs and respiratory system but also gastrointestinal (GI) tract, liver, pancreas, kidneys, heart, brain, and skin. Blood vessels are likely conduits for the virus dissemination to these distant organs. Importantly, ECs are also critical for vascular regeneration during injury/lesions healing and restoration of vascular network. The *World Journal of Gastroenterology* has published in last two years over 67 outstanding papers on COVID-19 infection with a focus on the GI tract, liver, pancreas, *etc.*, however, the role of the endothelial and vascular components as major targets for COVID-19-induced tissue injury, spreading to various organs, and injury healing have not been sufficiently emphasized. In the present article, we focus on these subjects and on current treatments including the most recent oral drugs molnupiravir and paxlovid that show a dramatic, significant efficacy in controlling severe COVID-19 infection.

Key Words: Endothelial cells; Impaired endothelial function; Blood vessels; SARS-CoV-2; COVID-19; Cytokine storm

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has enormous health care and economic impact on the entire world - infecting more than 250 million people in 213 countries and territories, causing death of more than 5 million (as of November 1, 2021). We comment here on some outstanding papers on COVID-19 published in *World Journal of Gastroenterology* and reviewed the important role of endothelium and blood vessels in COVID-19 infection. Endothelial cells and blood vessels are both the targets and a conduit for the spread of severe acute respiratory syndrome coronavirus 2 and play a critical role in COVID-19-induced tissue injury and dissemination to various organs. Pre-existing endothelial impaired function could make endothelial cells more sensitive to COVID-19 or at least COVID-19-induced impairment might be synergistic with pre-existing impairment. That could be one contributing factor explaining why older or diabetic patients have more severe responses to infection, since these conditions are already impacted impaired endothelial function.

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BIOGRAPHY

Andrzej S Tarnawski, MD, PhD, DSc (Med), AGAF, FACG: Received MD degree, PhD (pathology) and DSc (gastroenterology) from the University Medical School, Krakow, Poland, and became Associate Professor & V-Chair, Dept of Gastroenterology at that University. After completing GI fellowship at the University of Missouri, Columbia, MO, United States he joined the University of California, Irvine, USA as Associate Professor (1982-1986) and full Professor (1986-present). He served as: V-chair and Associate Chair, American Gastroenterological Association (AGA)/EGD 1997-1999 and 2008-2010; Scientific Director, Shimoda Symposia on Mucosal Defense in Japan (8 times); Chair, Research Fora DDW/AGA annual meetings (1996-2011); Chair, Pasteur Institute Euroconference and Chair/Co-chair of 68 other International Symposia. Publications, presentations & grants: 373 full, peer reviewed publications (*Lancet*, *Nature Med*, *JCI*, *Gastroenterology*, *Hepatology*, *Gut*, *PNAS*, *FASEB J*, *Am J Pathol*, *Cellular Mol Gastro Hepatol*, *Am J Physiol*, *Am J Gastroenterol*, *Endoscopy*, *Cell Signal*, *Cells*, and others); 20 book chapters; 533 presentations at international & U.S. meetings; 20 peer reviewed funded grants (NIH, VA Merit Review 1984-present), 4 US patents. Clinical and Research interest: Injury and protection of GI mucosa; cellular and molecular mechanisms of gastric, duodenal and esophageal healing-role of growth factors, signaling pathways, angiogenesis, NSAIDs, prostaglandins and *Helicobacter pylori* toxins; aging gastric mucosa; confocal endomicroscopy and molecular imaging; gene therapy. Awarded prestigious academic honors (*e.g.*, Glaxo Intl. Res. Award, Athalie-Clarke Award, Merentibus Medal Award, Peregrinator of Science Awards, Andre Robert's Distinguished Award, Notable Biomedical Research Investigator Award). Memberships: AGA (Fellow), Am. College of Gastroenterology (Fellow), Brit. Soc. of Gastroenterology, Japanese Soc. of Gastroenterology (Honorary), Hungarian Soc. of Gastroenterology (Honorary), Am. Soc. for Investigative Pathology, Association of Am. Physicians (by election) and others. Editorial Boards - 6 scientific journals. Sixteen of his former trainees hold academic positions in US Medical Schools (4 being Chairs of Departments). Twenty of his former international trainees and/or associates hold academic positions abroad (France, Germany, Hungary, Japan, Poland, Sweden, Switzerland) (Figure 1A).

Amrita Ahluwalia, PhD: Research Scientist and coronavirus disease 2019 (COVID-19) Investigator, Veterans Affairs Long Beach Healthcare System, Long Beach, United States. Awarded PhD (Physiology) by the Medical Sciences Program, Indiana University Bloomington, IN, United States. Publications/presentations/grants: 60 peer-reviewed research publications (*PNAS*, *Am J Physiol* , *Gene Therapy*, *Endocrinology*, *Molecular Endocrinology etc.*); 95 presentations at International and US research

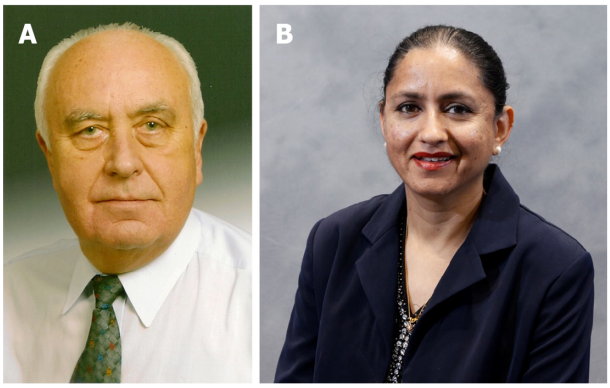


Figure 1 Authors. A: Andrzej S Tarnawski, Gastroenterology Research Department, University of California Irvine and the Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States; B: Amrita Ahluwalia, Research Service, Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States.

meetings; 6 peer-reviewed funded grants. Research Interests: Endothelial dysfunction, wound and tissue injury healing; gastroprotection; role of growth factors, angiogenesis, molecular imaging & gene therapy. Awards: Quest Diagnostics Young Investigator Award, AACR-AstraZeneca Scholar in Training Award, Robert W. Bullard Award - Outstanding Medical Science Student Award, Indiana University. Memberships: AGA, Am. Assoc. Physiology, Am. Heart Assoc.; Editorial Boards – 2 peer-reviewed scientific journals (Figure 1B).

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has had enormous health care and economic impact on the entire world - infecting more than 250 million people in 213 countries and territories, causing more than 5 million deaths (as of November 1, 2021). Its enormous magnitude is also reflected by an unprecedented number of publications related to COVID-19 so far approximate 210294 recorded in PubMed; 254358 recorded on PMC, and 3215 clinical trials just in 24 mo. These are staggering numbers compared to 47305 publications recorded on PubMed on *Helicobacter pylori (H. pylori)*– the world's most prevalent GI infection - published in about last 40 years.

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is highly infectious and transmitted by aerosol droplets. Therefore, it is not surprising that the respiratory tract including the lungs is the main affected organ by COVID-19 infection that leads to respiratory failure, hypoxia, multiorgan system failure and death. Numerous studies showed that COVID-19 not only affects the lungs and respiratory system but also the gastrointestinal tract (GI), liver, pancreas, kidneys, heart, brain, and skin[1-5]. SARS-CoV-2 RNA was detected in stool or rectal swabs in 34%-59% of infected patients[6]. The viral loads from stool samples peaked 2-3 wk after symptom onset and in some patients were detectable even after viral loads in the respiratory and/or sputum samples were not detectable[6]. The presence and persistence of viral RNA in the stool suggest the potential for enteric infection of SARS-CoV-2. This contention is supported by a study demonstrating that the GI tract is an alternative route for COVID-19 infection in the rhesus monkey model[7]. In that study, the authors showed that intranasal or gastric inoculation with SARS-CoV-2 induced infections and pathologic changes not only in respiratory tissues but also in digestive tissues[7]. In a recent letter to the *World Journal of Gastroenterology* (*WJG*) editor[8], Sica *et al*[8] contended that GI and hepatic involvement are the most common presenting symptoms of COVID-19 and multisystem inflammatory syndrome recently described in children and adolescents. This syndrome can lead to shock and multiple organ failure requiring intensive care[9].

Risk factors for COVID-19 severity include aging and comorbidities such as coronary artery disease, chronic kidney disease, hypertension, obesity, and diabetes [10-12], all of which exhibit preexisting endothelial dysfunction. However, the potential role of endothelial/vascular components as critical target sites for COVID-19-induced tissue injury and spreading to various organs, and the role of preexisting endothelial function impairment, *e.g.*, in aging or diabetes – conditions that facilitate

COVID-19 infection have not been sufficiently elaborated on. In the present article, we focus on these topics anticipating that providing a detailed information on endothelial cells (ECs) and vasculature in COVID-19 as critical targets may afford a better insight into the pathomechanism of this disease and add additional new therapies.

The SARS-CoV-2 virus spreads from its primary infection site (respiratory tract) to more distant organs indicating the involvement of ECs and blood vessels for disseminating infection. This contention is supported by some studies demonstrating the presence of SARS-CoV-2-like particles in ECs in several tissues *e.g.*, lung, kidneys, brain, and skin and observation that the clinical course of COVID-19 may include vascular complications such as thrombosis of blood vessels and thromboembolism[3,5,13-16].

The *WJG* has published in the last two years over 67 outstanding papers related to COVID-19 infection with a focus on GI tract and liver. These papers - original papers, retrospective studies and review articles on the pathophysiology, mechanisms, and clinical aspects and manifestations of COVID-19 related diseases of the digestive system including GI tubular system, liver, pancreas provided important information for the gastroenterologists, hepatologists, surgeons, researchers, pharmacologists, and clinicians. These papers provide information on the mechanisms of COVID-19 related tissue damage; the effects of immunosuppression in patients with inflammatory bowel disease and chronic liver disease; and the impact of COVID-19 on GI emergencies, endoscopy, diagnosis and treatments. These *WJG* articles were frequently viewed on the *WJG* website and cited in high-impact journals. We wish to point out one important paper by P. Samantha and AR Ghosh: "Environmental perspectives of COVID-19 outbreaks: A review" published in *World J Gastroenterol*. 2021 Sep 21;27(35):5822-585"[17]. In this paper the authors provided extensive information from an environmental perspective on the origin and current status of COVID-19[17] and summarized the geographical distribution of COVID-19 around the world including specific countries. They also elaborated on the details of coronavirus genus, species and receptors, virus susceptibility and incubation period, and summarized SARS-CoV-2 pathogenesis, the role of angiotensin-converting enzyme 2 (ACE2), the longevity of SARS-CoV-2 virus in the environment, meteorological influences, air quality and social impact. They emphasized that aging, cardiovascular diseases and diabetes predispose to COVID-19. The authors stressed that while drugs such as remdesivir, tocilizumab, lopinavir-ritonavir, azithromycin, *etc.*, are used in COVID-19 patients these drugs do not induce full recovery. The statement that there is no truly effective drug aimed at the causative agent, SARS-CoV-2 is no longer valid. On November 4 and 5, 2021 the released results of most recent clinical trials for COVID-19 treatments demonstrated that oral drugs inhibiting viral replication – Molnupiravir (Merck), and Paxlovid (Pfizer) showed very impressive efficacy in controlling severe COVID-19 infection. The interim analysis of the latter drug showed a dramatic approximate 90% reduction in risk of - hospitalization or death from COVID-19 compared to placebo in patients treated within three - five days of symptom onset. Most likely the vascular component of the disease was important part of this dramatic reduction.

Regarding COVID-19 pathomechanism, the potential role of endothelial and vascular components as critical target sites for COVID-19-induced tissue injury and spreading to various organs and the role of preexisting endothelial function impairment, *e.g.*, aging gastropathy has not been sufficiently emphasized. In this editorial article, we focus on the role vascular endothelium and blood vessels in COVID-19 infection (Table 1).

Increasing evidence suggests the essential role of endothelium and vasculature, in addition to the epithelial cells, in COVID-19 infection as a critical targets for SARS-CoV-2 and the resulting cytokine storm, and as the main effector for the pro-inflammatory and pro-coagulant state in COVID-19 patients[18,27-30]. Focus on ECs and vasculature in COVID-19 may also add additional insight into COVID-19 injury, its healing and tissue regeneration, and new therapies that impact endothelium and the blood vessels.

Although SARS-CoV-2 primarily targets the respiratory and alveolar epithelium, the high incidence of vascular complications in COVID-19 patients suggests that impaired function of ECs, which line the blood vessels and microvessels, may be critical factor in COVID-19 progression. SARS-CoV-2 causes endothelial dysfunction and thrombosis by two potential mechanisms: by directly infecting the endothelium, and disrupting its anti-thrombogenic and barrier properties, or indirectly by unleashing a local cytokine storm and systemic inflammatory response that results in endothelial injury (Table 2). Most likely, both these scenarios are in play in COVID-19.

Table 1 COVID-19 and endothelium/blood vessels

COVID-19 and endothelium/blood vessels
Endothelium and blood vessels are integral parts of COVID-19-induced tissue injury. Their injury is likely due to either direct viral infection and/or cytokine storm triggered by infection of adjacent epithelial cells and inflammatory response[18].
Blood vessels are critical for virus dissemination to distant organs.
Preexisting-impaired endothelial function, *e.g.*, in aging or diabetes are likely predisposing factors COVID-19. Our studies demonstrated that aging gastric mucosa has increased susceptibility to injury and prominent EC abnormalities (decreased VEGF, NGF and impaired mitochondrial function)[19-21].
ECs are critical for vascular regeneration (through angiogenesis and vasculogenesis) during injury/lesions healing and therefore are essential for the delivery of oxygen and nutrients to the healing site[22,23].
Several growth factors *e.g.*, NGF, IGF-1, HGF and BMD-stem cells may facilitate tissue regeneration in the healing phase[20,24,25].
Long-term effects of COVID-19, its vaccines and treatment on endothelium and vasculature remain to be determined.
Recently, new oral drugs inhibiting viral replication–Molnupiravir (Merck) and Paxlovid (Pfizer) showed significant efficacy in controlling severe COVID-19 infection by inhibiting viral replication. The interim analysis of the latter drug showed an 89% reduction in risk of COVID-19-related hospitalization or death from any cause compared to placebo in patients treated within three-five days of symptom onset[26].

COVID-19: Coronavirus disease 2019; EC: Endothelial cell; VEGF: Vascular endothelial growth factor; NGF: Nerve growth factor.

Table 2 Scenarios by which SARS-CoV-2 elicits endothelial damage

Scenario A: SARS-CoV-2 infection	Scenario B: Cytokine storm
SARS-CoV-2 infects and replicates within vascular ECs and new virus particles are released into the blood vessel. These virions can infect neighboring cells or are carried to distant organs *via* circulation	↑ IL-6, IL-1β, and TNFα release (cytokine storm) → endothelial damage ↑ vascular permeability → plasma extravasation ↑ vWF & FVIII (promote clot formation) and ↑ PAI-1 (inhibits clots lysis) → hypercoagulation

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin; TNF: Tumor necrosis factor; vWF: von Willebrand factor.

Endothelium in normal and pathological conditions. Role in homeostasis, tissue repair and healing

The endothelium is a key player in vascular homeostasis[29,31-33]. ECs are critical for supplying oxygen and other nutrients to all cells and tissues, and are involved in coagulation and the generation of vasoactive substances, prostanoids, hormones and growth factors[33-38]. The unstimulated vascular endothelium is normally impermeable and acts as a selective barrier regulating exchange of fluids, nutrient delivery and waste removal while preventing entry of pathogens and harmful substances into the tissues. Microvessels consist of a single layer of thin (approximate 0.5-1 μm) ECs and occasional adherent cells such as pericytes[34-38]. The endothelial "barrier between neighboring ECs formed by prominent tight junctions prevents diffusion between cells. ECs act as a barrier between blood and the interstitial tissue, and regulate various physiological processes such as angiogenesis, inflammation, and immune response[31,35,36]. The endothelium contains special vesicles - Weibel-Palade bodies, which store various factors that regulate blood coagulation and leukocyte recruitment and extravasation such as von Willebrand factor (vWF), P-selectin, chemokines, interleukin-8, and eotaxin-3; endothelin-1, angiopoietin-2 and osteoprotegerin[39-42].

In response to local stimuli, ECs secrete endothelin and leukotriene C4 (potent vasoconstrictors), nitric oxide (NO) and prostacyclin (PGI2) (vasodilators) and empty the contents of the Weibel-Palade vesicles that affect the tone of vascular smooth muscle and result in neutrophil adhesion and/or other autocrine and/or paracrine actions. NO, prostacyclin, prostaglandin E2 (PGE2), carbon monoxide (CO), tissue plasminogen activator, vascular endothelial growth factor (VEGF) and bFGF are endothelial mediators that reduce platelet and leukocyte activation, prevent thrombi formation, promote thrombolysis, maintain tissue perfusion, and protect the microvascular wall against acute damage[33,36-38,43-46]. For example, our previous study demonstrated that 16,16 dimethyl PGE2 protects human gastric mucosa against injury by 40% ethanol by protecting and preserving integrity of endothelial cells of

gastric microvessels[47]. In response to wounding, infections or injurious stimuli, attachment between ECs is lost, resulting in increased endothelial permeability and edema[48].

The endothelium and blood vessels are integral parts of any tissue injury including COVID-19. Our previous studies demonstrated that ECs are critical targets of gastric mucosal injury by NSAIDs and ethanol, they initiate angiogenesis, and that age-related endothelial dysfunction of human and rat gastric endothelial cells results in impaired angiogenesis and delayed healing[19,20,24]. Our studies on aging gastropathy showed aging-related defects in ECs functions - angiogenesis, cell migration, proliferation, and healing of injury[19-21,49]. In a recent study, we also showed the critical role of mitochondria in aging gastric ECs; aging ECs have fewer mitochondria, and reduced mitochondrial membrane potential[50] that result in reduced ATP generation (Figure 2). We also demonstrated that treatment with VEGF and nerve growth factor (NGF) restores angiogenesis in cultured aging gastric ECs [20], accelerates healing of gastric ulcers and improves the quality of mucosal regeneration *in vivo* in aging rats[20,24].

Endothelial cells and COVID-19

SARS-CoV-2 is a single, positive-stranded RNA virus that uses a spike-protein (S-protein) expressed on its envelope to bind to the host cell's human protein receptor ACE2[51-53]. The human ACE2 protein was initially identified as ACE-related carboxypeptidase membrane-associated and secreted enzyme expressed predominantly on the endothelium of the human heart, kidney, and testis [54]. However, it is widely expressed in various cells and tissues[55]. SARS-CoV-2 employs the ACE2 receptor, transmembrane serine protease 2 (TMPRSS-2), and cathepsin B and L (CTSB, and CTSL) for infection[51-53,56,57]. SARS-CoV-2 was detected in the respiratory tract, kidneys, liver, heart, and brain (all of which are highly vascularized tissues) of infected individuals[55]. ECs, which line the blood vessels of all organs and maintain microvascular integrity, express the ACE2 receptor and the cellular proteases TMPRSS-2, CTSB, and CTSL[57]. ECs are, therefore, a target for SARS-CoV-2 and blood vessels likely route of this virus dissemination to various organs. Electron microscopy (EM) and histologic studies detected SARS-CoV-2 virus-like particles and proteins in ECs of the kidney, small bowel, lung, myocardium, skin, and brain[3,5,13-16]. Ackerman *et al*[15] showed abnormalities within the pulmonary microvasculature with congestion and micro-thrombi in lungs of COVID-19 patients, and visualized endothelial injury and lumen filled with cell fragments and degenerated organelles by electron microscopy. That study also showed increased ACE2-positive ECs and significant changes in endothelial morphology in lung autopsies of COVID-19 patients [15]. Varga *et al*[5] using EM evaluation reported evidence of viral particles in renal ECs of COVID-19 patients presenting with endotheliitis, which is an immune and inflammatory response within the endothelium of blood vessels.

Other studies visualized SARS-CoV-2 proteins in dermal and renal endothelium[13,58]. While some studies were not able to corroborate presence of SARS-CoV-2 in ECs of some tissues, there is strong evidence to support that SARS-CoV-2 infects ECs. Monteil *et al*[59] demonstrated that SARS-CoV-2 infects blood vessel organoids. SARS-CoV-2 virus particles range from approximate 70 to 120 nm[60-63]; therefore, in the absence of preexisting tissue injury, the virus would need to pass through the ECs to infect other tissues.

Endothelial dysfunction

The term endothelial dysfunction was originally used to identify the shift from a normal quiescent endothelium to an impaired endothelium with the inability to generate nitric oxide and other vasodilators. In a broader definition, endothelial dysfunction includes impairment of endothelial function (that we used for aging endothelium in our previous papers) - reduced angiogenesis, pro-inflammatory, pro-vasoconstriction, proliferative, and pro-coagulant phenotype[18,64-66]. In certain pathological conditions characterized by preexisting endothelial dysfunction, the ACE/Ang II axis is upregulated resulting in vasoconstriction, thrombosis, fibrosis, coagulopathy, and thrombophilia.

Endothelial dysfunction and endotheliitis in COVID-19

Emerging evidence indicates that preexisting endothelial dysfunction predisposes to COVID-19 infection and that COVID-19 induced endotheliitis further impairs endothelial integrity and function[27-30,32,34,67-76]. This is evidenced by the critical role of vascular endothelium in inflammation that results in dysregulation of cytokines

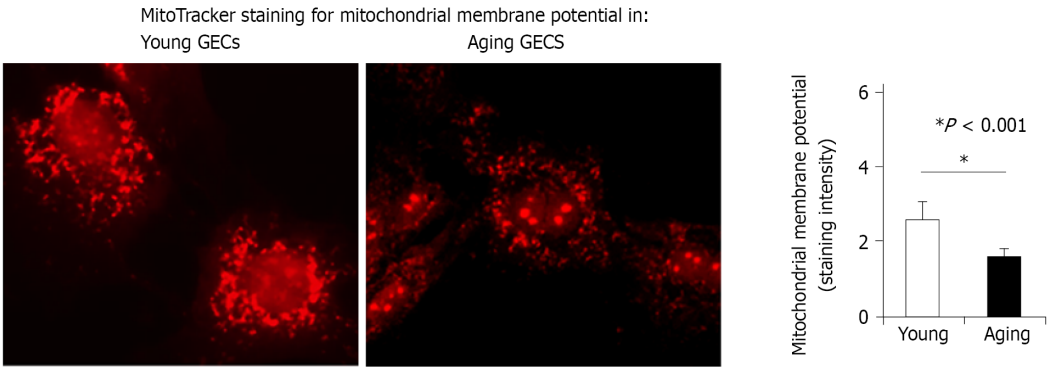


Figure 2 Mitotracker staining for mitochondrial membrane potential in young gastric endothelial cells and aging gastric endothelial cells. Aging gastric endothelial cells (GECs) have significantly reduced mtMP reflecting impaired mitochondrial function *vs* young GECs [reproduced with permission from reference[20], which is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)]. GECs: Gastric endothelial cells.

in acute respiratory distress syndrome as well as multiple cardiovascular pathologies [18,27,30,32,64,71,73]. The ubiquitous expression of ACE-2 on ECs in all tissues suggests that SARS-CoV-2 can spread *via* circulation throughout the body and affect multiple organs[55].

The sequential steps of SARS-CoV-2 infection of ECs that result in endothelial pathology and a procoagulant, hypofibrinolytic state of the endothelium are summarized in Figure 3. SARS-CoV-2 utilizes the ACE2 receptors and cellular proteases (TMPRSS-2, CTSB and CTSL) infect the host cells including ECs[51-53,56, 57]. The virus then replicates within the cells and is released into the blood vessels, which then disseminate the virus to distant organs. Severe COVID-19 results in increased production of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) which is referred to as cytokine storm[29,77,78]. The binding of IL-6 to its receptors on ECs increases vascular permeability, induces capillary leakage, and unleashes a cytokine storm by further increasing the secretion of IL-6, IL-8, and MCP-1 by ECs[29,70,78]. The cytokine storm in COVID-19 patients exposes the endothelium to pro-inflammatory cytokines resulting in leukocyte recruitment and inflammation and can lead to EC death that contributes to increased vascular permeability and end-organ damage[18,29]. In addition, activated ECs produce increased amounts of vWF and factor VIII, which participate in clot formation thereby inducing a pro-coagulant state. Furthermore, ECs produce increased amounts of PAI-1 that inhibits the degradation of clots and induces a hypofibrinolytic state[29,70,78].

The initial SARS-CoV-2 infection and vascular damage in pulmonary tissues can result in the release of ECs into the circulation. Increased numbers of circulating ECs (CECs) have been demonstrated in conditions associated with vascular damage[79-82]. Increased CECs may potentiate the spread to distant extrapulmonary tissues. Numerous extrapulmonary manifestations of SARS-CoV-2 infection such as acute kidney injury, thrombotic complications, myocardial dysfunction and arrhythmia, heart failure, venous thromboembolism, GI symptoms, hepatocellular injury, neurologic illnesses, ocular symptoms, and dermatologic complications have been documented[1]. Endothelial injury may be the underlying mechanism for both pulmonary and extrapulmonary manifestations of COVID-19.

Endothelial cells are critical for vascular regeneration through angiogenesis and vasculogenesis during the injury/lesions healing phase

The process of tissue injury healing involves tissue and vascular regeneration[32,34,75, 83,84]. The latter is mediated by the sprouting of ECs from pre-existing vessels from areas bordering injury (angiogenesis), or the formation of new blood vessels from bone marrow-derived angiogenic precursor cells (vasculogenesis)[22,23,85]. Blood vessel reconstruction is regulated by angiogenic growth factors and involves the activation of genes such as basic fibroblast growth factor (bFGF or FGF-2) and its receptors; VEGF and its receptor; angiopoietins -Ang 1 and Ang 2, and their receptor, COX-2, serum response factor, NGF, stromal-derived factor 1[25]. Our previous studies demonstrated the aging-related decrease in the expression of VEGF and NGF in ECs and that treatment with VEGF and NGF restore angiogenesis in aging gastric ECs (Figure 4)[20, 21]. Furthermore, we showed that local NGF therapy of gastric ulcers increased

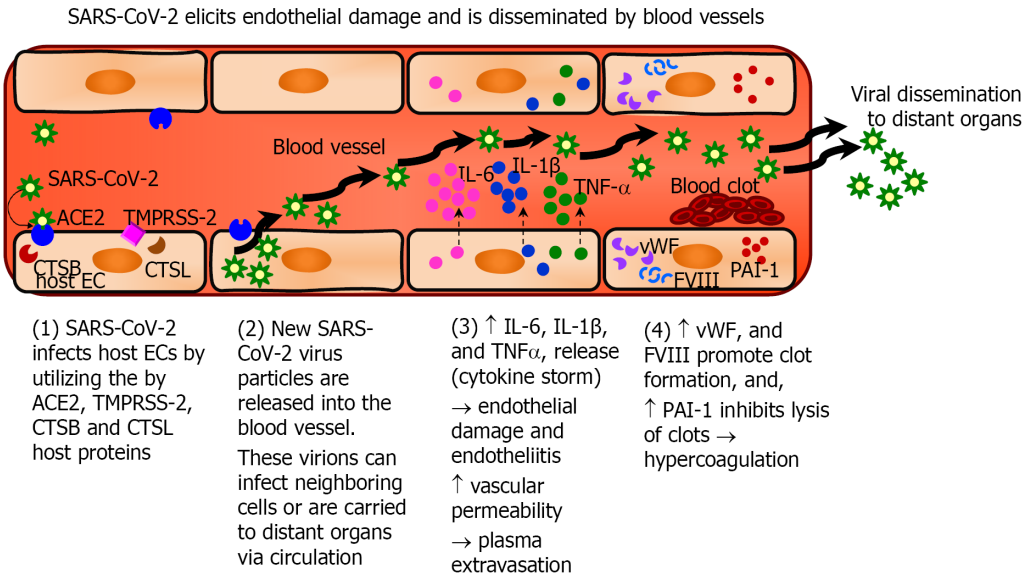


Figure 3 Sequential steps of SARS-CoV-2 infection of endothelial cells and endothelial damage. SARS-CoV-2 infects endothelial cells (ECs) using the host angiotensin-converting enzyme 2 receptors and cellular proteases (transmembrane serine protease 2, and cathepsin B and L). The virus then replicates within the cells and is released into the blood vessels, which then disseminate the virus to distant organs. Severe COVID-19 results in a cytokine storm wherein there is increased production of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1, interleukin-1 β (IL-1 β) and tumor necrosis factor-α (TNF-α), and results in endothelial damage and endotheliitis, and demonstrated increased vascular permeability that cause plasma extravasation. Activated ECs produce increased amounts of vWF and factor VIII, and PAI-1, which induce a pro-coagulant, hypofibrinolytic state. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ECs: Endothelial cells; ACE2: Angiotensin-converting enzyme 2; TMPRSS-2: Transmembrane serine protease 2; CTSB: Cathepsin B; CTSL: Cathepsin L; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

angiogenesis, promoted revascularization, and accelerated gastric ulcer healing in aging rats[20].

The long-term effects of COVID-19 and its vaccines on endothelium and vasculature remains unknown

SARS-CoV-2 infection was first reported in 2019 and rapid, breakthrough research resulted in the development of several effective COVID-19 vaccines. Although these vaccines have proven effective in reducing the infection and severity of COVID-19, the long-term effects of the disease and the vaccines on ECs and blood vasculature are still to be determined.

POTENTIAL TREATMENTS

Two recent outstanding studies published by the Baishideng Publishing Group in the *World Journal of Virology* outlined the current therapies that have been utilized in COVID-19 treatment[86,87]. We wish to add to this list additional investigational treatments in ongoing clinical trials (Table 3) and describe two additional oral drugs that were announced in early November 2021 as potential COVID-19 treatments Molnupiravir (Merck) and Paxlovid (PF-07321332).

During recent press releases two newest oral drugs inhibiting SARS-CoV-2 replication were recently presented. Are they game changers? On November 4 and 5, 2021 two oral drugs were announced as novel COVID-19 treatments - Molnupiravir (Merck) and Paxlovid (PF-07321332). Both these drugs showed dramatic efficacy in controlling severe COVID-19 infection. The oral drug Molnupiravir (EIDD-2801) was developed by US-based Merck & Co Inc and Ridgeback Biotherapeutics[88] and investigated in a clinical trial (NCT04405570) to eliminate SARS-CoV-2 virus load in infected patients, has since been approved in the UK to treat patients with mild to moderate COVID-19 and at least one risk factor such as older age, diabetes, obesity, and heart disease that predisposes them for developing severe illness. Molnupiravir is the prodrug of the ribonucleoside analog β-D-N4-hydroxycytidine and is rapidly converted by host kinases in plasma to the active 5′-triphosphate form. The latter is a competitive substrate for SARS-CoV-2 RNA-dependent RNA polymerase and causes mutations in the viral genome during replication that makes the virus non-viable. The

Table 3 Summary of the investigational interventions/treatments for COVID-19 in clinical trials

Intervention/ Treatment	Mode of action	Dose	Route	ClinicalTrials.gov Identifier
Ronapreve/REGN-COV2 (REGN10933 and REGN10987)	Monoclonal antibodies against spike proteins	8 g once, or 4 g twice	IV	NCT04425629
Lopinavir/Ritonavir	Inhibitor of the HIV protease and cytochrome P-450 CYP3A	200/ 50 mg; (4 tablets twice a day on day 1 followed by 2 tablets twice a day for 9 d)	Oral	NCT04403100
Remdesivir (RDV, GS-5734, Veklury)	Inhibitor of RNA-dependent RNA polymerase	200 mg on day 1 followed by 100 mg for 4-9 d	IV	NCT04292899
Hyperimmune Plasma (COV19-PLASMA)	Immunotherapy	250-300 mL up to 3 times over 5 d	IV	NCT04321421
Tocilizumab (TCZ, ROACTEMRA)	Humanized anti-IL6 receptor monoclonal antibody	8 mg/kg single infusion, up to 800 mg	IV	NCT04320615
Sarilumab (Kevzara, REGN88, SAR153191)	Monoclonal antibody against IL-6 receptor alpha	200 mg or 400 mg; single dose and multiple doses	IV	NCT04315298
Anakinra (KINERET)	Monoclonal antibody against the IL-1 receptor	100 mg daily up to 28 d	SC	NCT04330638
Siltuximab (SYLVANT)	Chimeric anti-IL-6 antibody	11 mg/kg single infusion	IV	NCT04330638
Eculizumab	Monoclonal antibody against complement protein C5	900 mg every 7 d	IV	NCT04288713
Methyl-prednisolone (MP)	Immunosuppression against cytokine storm	80 mg/kg IV bolus, followed by infusion of 80 mg/d for at least 8 d and then oral MP 16 mg or 20 mg IV twice daily	Oral-IV	NCT04323592
Heparin	Antithrombotic agents	10 units/kg/h	IV	NCT04367831
Enoxaparin (Lovenox)	Antithrombotic agents	1 mg/kg	SC	NCT04367831
Dexamethasone	Immunosuppression against cytokine storm	20 mg/d (5 d) then 10 mg/d (5 d)	IV	NCT04325061
Vitamin C	Antioxidant	12 g infusion twice a day for 7 d	IV	NCT04264533
Melatonin	Antioxidant	3 or 30 mg three times a day for 14 d	Oral	NCT04784754
CoQ10	Antioxidant	500 mg/day for 6 wk	Oral	NCT04960215

IL.: Interleukin.

specific action of this drug on SARS-CoV-2 infection of ECs is not known.

The second drug, Paxlovid (PF-07321332; ritonavir) is a SARS-CoV-2 protease inhibitor antiviral therapy[26]. PF-07321332 is an inhibitor of the SARS-CoV-2 3-chymotrypsin-like cysteine protease that is essential for SARS-CoV-2 replication[26, 89]. Ritonavir is a protease inhibitor that slows down the metabolism/breakdown and therefore, increasing the bioavailability of other protease inhibitors including PF-07321332 in the body[90]. Studies published on November 2, 2021, in Science reported the discovery and characterization of PF-07321332 (Paxlovid)[26]. These studies demonstrated that Paxlovid inhibits SARS-CoV-2 replication *in vitro* in human adenocarcinoma-derived alveolar basal epithelial and differentiated normal human bronchial epithelial cells[26]. This drug showed *in vitro* coronavirus antiviral activity against all coronaviruses infecting humans and excellent off-target selectivity and *in vivo* safety profiles.

That study also showed the efficacy of orally administered 300 or 1000 mg/kg PF-07321332 against SARS-CoV-2 infection *in vivo* in a mouse model challenged intranasally with SARS-CoV-2 MA10 (CCID50). PF-07321332 Limited cellular infiltration by SARS-CoV-2 and protected lung tissue from damage compared to placebo treatment in that study[26]. Most importantly, the interim analysis of the Paxlovid human clinical trial demonstrated a dramatic approximate 90% reduction in COVID-19-related hospitalization or death in high-risk patients treated within 3 to 5 d of symptom onset compared to placebo. Since this drug inhibits virus replication the chance of endothelial infection and dissemination of virus *via* blood vessel is reduced. We postulate that ECs and blood vessels are likely an important part of this drug's clinical efficacy. Naturally, this contention requires further careful analysis and confirmation, and in-depth insight, since the biological effects of these drugs are

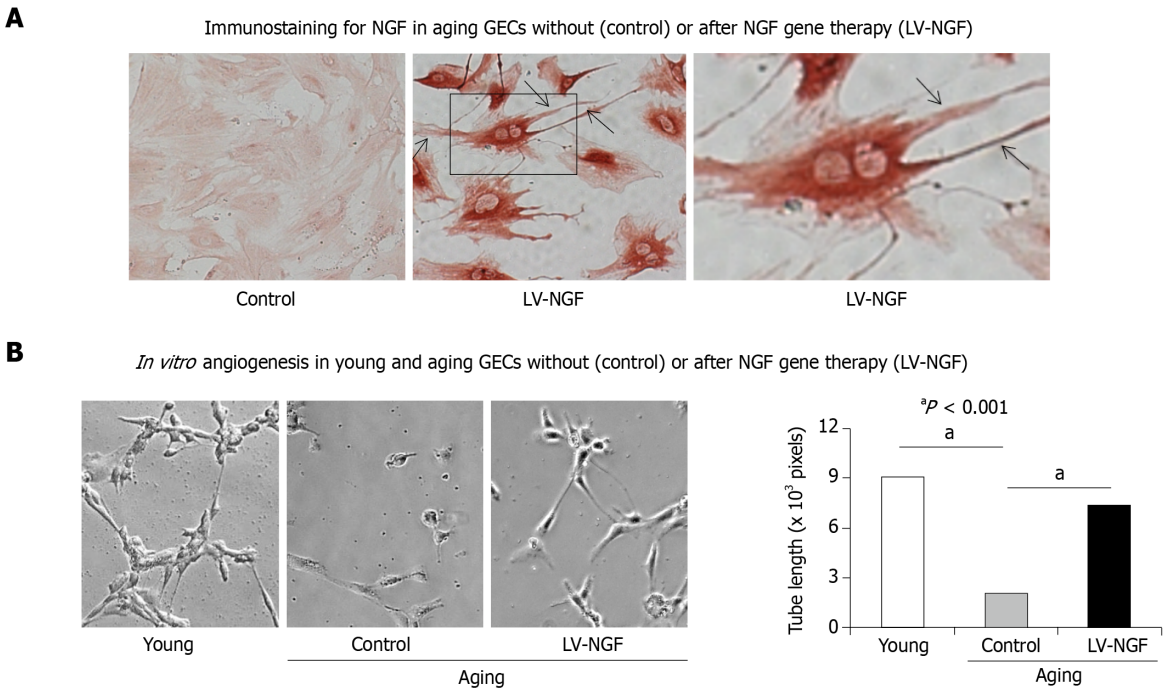


Figure 4 Nerve growth factor gene therapy increases nerve growth factor expression and reverses impaired *in vitro* angiogenesis in aging gastric endothelial cells. A: Nerve growth factor (NGF) gene therapy of aging Gastric endothelial cells (GECs) using lentiviral-NGF (LV-NGF) induced NGF expression (brown staining) and extensive, long filopodia (arrows) reflecting a change in these cells to an angiogenic phenotype; aging GECs without gene therapy (negative controls) have minimal NGF expression and lack filopodia; B: NGF gene therapy with LV-NGF resulted in 3.7-fold increased *in vitro* angiogenesis at 6 h in aging GECs *vs* negative controls (control). Panels are representative images of capillary-like tube formation. Original magnification: × 200. Data are means ± SD (n = 6). ($^aP < 0.001$). NGF: Nerve growth factor; GEC: Gastric endothelial cells; LV: Lentiviral. Reproduced with permission from reference[20], which is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

largely unknown[91,92]. This sentiment and discussion regarding these oral drugs are summarized in the November 10, 2021 Nature article titled COVID antiviral pills: what scientists still want to know[91]. On December 22, 2021, the US Food and Drug Administration issued an emergency use authorization of Paxlovid to treat mild and moderate COVID-19 (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19).

Other drugs that may be repurposed for COVID-19 treatment include melatonin, coenzyme Q 10 (CoQ10). Melatonin with its anti-inflammatory and anti-oxidative effects can protect against bacterial and viral infections[93-95] and an ongoing clinical study is investigating the efficacy of melatonin in COVID-19 (NCT: 04784754). A clinical trial is investigating the effect of high-dose CoQ10 in long-term COVID-19 patients (NCT: 04960215). The use of growth factors - VEGF, NGF, EGF and KGF, and treatment with adipose-derived stem cells (ADSCs) may be useful for COVID-19 therapy in both the initial and especially the regenerative, healing phase of the disease. A recent study demonstrated that ADSCs release exosomes that secrete various growth factors such as NGF, IGF1, HGF, *etc*.) that may alleviate the cytokine storm in COVID-19 patients[96].

CONCLUSION

While respiratory epithelium is the main route of virus entry, the ECs lining blood vessels are an integral part of COVID-19 disease progression and multi-organ spread. COVID-19 not only affects the lungs and respiratory system but also gastrointestinal tract, liver, pancreas, kidneys, heart, brain, and skin. Blood vessels serve as conduits for the virus dissemination to these distant organs. Importantly, ECs are also critical for vascular regeneration during injury/lesions healing and restoration of vascular network. In the present article, we reviewed the role of the endothelial and vascular components as major targets for COVID-19-induced tissue injury, spreading to various organs, and injury healing, and the current treatments for COVID-19 including the most recent oral drugs Molnupiravir and Paxlovid.

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I dedicate this article to my awesome wife Hella Gergely Tarnawski, who encouraged me to write this article and has been my inspiration. Tarnawski A.S.

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FRONTIER

Eliminating viral hepatitis in children after liver transplants: How to reach the goal by 2030

Palittiya Sintusek, Kessarin Thanapirom, Piyawat Komolmit, Yong Poovorawan

ORCID number: Palittiya Sintusek 0000-0003-4441-0151; Kessarin Thanapirom 0000-0003-2333-1702; Piyawat Komolmit 0000-0002-1357-9547; Yong Poovorawan 0000-0002-2337-6807.

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Country/Territory of origin: Thailand

Palittiya Sintusek, The Thai Pediatric Gastroenterology, Hepatology and Immunology (TPGHAI) Research Unit, Chulalongkorn University, Bangkok 10330, Thailand

Palittiya Sintusek, Division of Gastroenterology, Department of Pediatrics, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok 10330, Thailand

Kessarin Thanapirom, Piyawat Komolmit, Division of Gastroenterology, Department of Medicine, Liver Fibrosis and Cirrhosis Research Unit, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand

Kessarin Thanapirom, Piyawat Komolmit, Center of Excellence in Liver Diseases, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok 10330, Thailand

Yong Poovorawan, Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Corresponding author: Yong Poovorawan, MD, Professor, Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Pathumwan District, Bangkok 10330, Thailand. yong.p@chula.ac.th

Abstract

Viral hepatitis infections are a great burden in children who have received liver transplant. Hepatotropic viruses can cause liver inflammation that can develop into liver graft fibrosis and cirrhosis over the long term. Immunological reactions due to viral hepatitis infections are associated with or can mimic graft rejection, rendering the condition difficult to manage. Prevention strategies using vaccinations are agreeable to patients, safe, cost-effective and practical. Hence, strategies to eliminate viral hepatitis A and B focus mainly on immunization programmes for children who have received a liver transplant. Although a vaccine has been developed to prevent hepatitis C and E viruses, its use is not licensed worldwide. Consequently, eliminating hepatitis C and E viruses mainly involves early detection in children with suspected cases and effective treatment with antiviral therapy. Good hygiene and sanitation are also important to prevent hepatitis A and E infections. Donor blood products and liver grafts should be screened for hepatitis B, C and E in children who are undergoing liver transplantation. Future research on early detection of viral hepatitis infections should include molecular techniques for detecting hepatitis B and E. Moreover, novel antiviral drugs for eradicating viral hepatitis that are highly effective and safe are needed for

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children who have undergone liver transplantation.

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Core Tip: Viral hepatitis infections are a great burden for pediatric liver transplant recipients. Strategies to prevent infection include immunization, good sanitation and screening donor blood products and liver grafts for hepatitis B, C and E. In children infected with viral hepatitis who have received a liver transplant, early detection is crucial to guide proper management, as the infection can mimic or cause graft rejection. Effective antiviral therapy should be initiated when treating children with hepatitis B and C. Patients infected with hepatitis B who have undergone successful viral eradication should be revaccinated to maintain high hepatitis B surface antibodies to guarantee immunoprotection.

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INTRODUCTION

Viral hepatitis is an infectious disease leading to high morbidity and mortality, especially in endemic areas such as Asia. Hepatitis viruses are hepatotropic and are classified into types A, B, C, D and E. In immunocompromised patients, including children who have undergone liver transplantation (LT) and typically receive lifelong immunosuppressants, nearly all viral hepatitis infections are chronic, progressing to liver fibrosis and cirrhosis in the long term. Hepatitis A is the only hepatitis virus that presents as an acute self-limiting infection but that is more severe in immunocompromised patients than in healthy individuals. Because viral hepatitis places a heavy burden on patients, strategies for prevention, early detection and prompt, effective management are crucial for graft survival and long-term outcomes in children after LT. In this review, we focus on lessons learned and future opportunities to develop effective strategies to eliminate hepatitis A, B, C and E in children after LT.

BIOGRAPHY

Yong Poovorawan (Figure 1), MD is currently the Professor and the head of the Center of Excellence in Clinical Virology at the Faculty of Medicine, Chulalongkorn University, Bangkok. Professor Poovorawan obtained the medical degree in 1974 and his specialization in paediatrics in 1978 from King Chulalongkorn Memorial Hospital, Chulalongkorn University. In 1984, he became a research fellow on the field of paediatric hepatology at King's College Hospital Medical School, London. Professor Poovorawan has been working in the Department of Pediatrics at Chulalongkorn University, beginning as a lecturer and becoming Professor in 1991. Professor Poovorawan has received many research awards and honours, including the Outstanding Researcher Award in 1997 from the National Research Council of Thailand, Outstanding Scientist Award in 1997 from the Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King, Mahidol University-B-Braun Award in 2002, Thailand Research Fund Award in 2004 and has been nominated Senior Research Scholar by the Thailand Research Fund since 1997. He also received the Outstanding Best Teachers Award in 2004 from the Thailand National University Teacher Association. He is a leader who has been working on Viral hepatitis in Thailand. Outstanding Prof. Thailand Research Fund (2012-2014), Research Chair Grant, NSTDA (2014), Outstanding Achievement Doctor from the



Figure 1 Yong Poovorawan, MD, Professor, Excellence Center of Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Medical Council of Thailand (2018), Achievement Award in Virology, Genetics Society of Thailand (2018), Achievement Award from the National Vaccine Institute of Thailand (2019). His work on avian influenza in Thailand also received outstanding research awards from the Thailand Research Fund in 2004 and the National Research Council in 2006. He is a member of the expanded programme on immunization vaccine, viral hepatitis and emerging diseases of the Center Disease Control, Ministry of Public Health. Professor Poovorawan has authored and co-authored more than 614 publications in the fields of hepatitis, paediatric hepatology and virology, with H-index 66 on Google Scholar.

HEPATITIS A VIRUS

Manifestations of hepatitis A virus (HAV) infection mainly derive from the immunologic responses to the virus and might present as severe acute liver failure in healthy children[1]. The incidence of fulminant hepatic failure from HAV infection varies from 0.1% to 1% but increases in patients with chronic liver disease[2-4]. HAV is rarely reported in children after LT. In our centre, no children have been admitted with HAV after LT[5], likely because Thailand has significantly decreased the HAV infection rate in past decades[6]. However, HAV can mimic graft rejection or presents as recurrent HAV post-LT[7]. Immunization is the mainstay of prevention and should be given before LT. Short- and long-term studies (ranging from 2-48 mo) of immunologic responses to HAV vaccines in adults post-LT have revealed variable seroprotection rates ranging from 26%-97% after 2 doses of the vaccine[8-11]. In a 4-wk assessment, Ferreira *et al*[12] compared HAV vaccine immunogenicity between children with chronic liver disease and healthy controls and found that 97% of the former and 100% of the latter showed seroconversion, with geometric mean titres of 812.4 mIU/mL and 2344.90 mIU/mL, respectively, after 2 doses of the HAV vaccine[12]. However, no study has reported HAV vaccine immunogenicity in children post-LT. Arslan *et al*[13] found that 18% and 29% of adult patients lost humoral immunity to HAV at 1 and 2 years, respectively, post-LT. Interestingly, one case study reported a 55-year-old man who was previously immunized and was HAV-IgG-positive but had an acute HAV infection in-hospital post-LT[14]. This case report suggested that rapid HAV seroconversion after LT should be regularly monitored and that revaccination should be considered for patients with a loss of HAV immunity. We recommend providing HAV vaccines to all children waiting for LT, as the humoral immune response to the HAV vaccine is favourable[12]. Nevertheless, HAV vaccines are provided only for children older than 1 year, and younger children may require LT before being eligible for the HAV vaccine. Hence, post-LT HAV immunization is needed. Further studies should be conducted regarding long-term immunologic responses of HAV to confirm the efficacy of 2-dose HAV vaccines in immunocompromised children. Apart from the vaccine, other necessary strategies include improving sanitation and avoiding uncooked food.

HEPATITIS B VIRUS

Immunization: A public health weapon for preventing hepatitis B virus infection

Pre-LT: Since universal hepatitis B virus (HBV) vaccination programmes began in the 1990s[15], HBV infection prevalence has rapidly decreased worldwide. HBV vaccine series that include vaccines at birth, 1-2 and 6-12 mo can reduce mother-to-child transmission, the major mode of HBV transmission in children, from 65%-90% to 3.6%-4.0%[16,17]. Indeed, the seroprotective rate after a complete HBV series is > 95%[18]. In our cohort study, although seroprotective rates decreased to 44% over a 20-year follow-up, 93.1% of the children exhibited seroconversion after a booster dose[19]. The presence of immune memory cells after the booster dose confirmed waning immunity with an anamnestic response, indicating increased levels of hepatitis B surface antibodies (anti-HBs)[19]. However, in immunocompromised patients with chronic liver diseases or cirrhosis, revaccination yields unsatisfactory outcomes, with seroconversion rates of 37.0%-90.9% on conventional schedules[20-28] and 16%-72% on accelerated/super-accelerated[29-37] schedules. Many studies on HBV schedules have been conducted to improve immunologic responses after revaccination in nonresponders, mainly using adult data, with different doses, routes, vaccine types, numbers and injection intervals. Regardless, no differences in the efficacy of these regimens have been shown[38]. Overall, time is a concern for participants awaiting LT, and super-accelerated or accelerated regimens should be considered for short-term prevention of HBV infections during and after LT[39].

During LT: External sources of HBV transmission, such as blood products, medical instruments and transmission by hospital personnel or close contacts, are concerns. Anti-HBs may decline after excessive plasma loss during surgery, and occult HBV infections from positive hepatitis B core antibody (anti-HBc) blood products have been reported[40].

Post-LT: Immunologic loss of HBV is common after LT [41], and *de novo* hepatitis B infection (DNH) was observed in our paediatric LT centre[42]. DNH is likely related to acquired HBV infections from endemic environments or from HBV reactivation from positive anti-HBc allografts during immunologic loss[43-47]. In our centre, the anti-HBs loss rate increased rapidly after LT, and 46%, 57% and 82% of patients had anti-HBs levels of < 10 mIU/mL at 1 year, 2 years and > 3 years after LT, respectively. One case of DNH was detected at 3 years after LT, though anti-HBs levels were > 1000 mIU/mL before LT[42]. Hence, regular monitoring for anti-HBs and revaccination after LT are crucial. Studies of immunogenicity to HBV revaccination after LT have reported higher humoral immune responses in children than in adults (up to 100% *vs* 33.3%-63.8%); however, immunity waned, and the patients needed frequent booster doses to maintain high seroprotective levels[43]. In healthy adults not responding to conventional vaccine schedules, a systematic review found no differences in seroconversion rates according to dosage or vaccine administration route[38]. However, to date, no study has been conducted involving children in this population. We conducted studies of immunologic responses to standard *vs* double-dose HBV vaccine series (at 0, 1 and 6 mo) in children after LT exhibiting anti-HBs loss and found response rates of 91.6% and 85% after a 6-mo follow-up, with no statistically significant difference in anti-HBs level between the two regimens (unpublished data). Hence, short-term assessment revealed that HBV revaccinations in children after LT are highly effective and safe.

Positive anti-HBc allografts are considered a major risk factor of DNH after LT, especially in patients without prior seroprotection or rapid anti-HBs loss after LT. In addition to being revaccinated 3-6 mo after LT, other strategies to prevent DNH include antiviral therapy and/or passive immunity with hepatitis B immunoglobulin (HBIG). Unlike the many studies that have used adult data and investigated several strategies, few studies of prophylactic strategies against DNH have been conducted in children who receive positive anti-HBc allografts[43,44,48-51]. Song *et al*[48] reported the efficacy of pre- and post-LT HBV vaccinations to prevent DNH and recommended a prophylactic strategy to maintain anti-HBs levels at ≥ 1000 mIU/mL pre-LT and ≥ 200 mIU/mL post-LT without antiviral consideration. The DNH rate when using this strategy was 1.3%[48]. However, anti-HBs levels may rapidly decline after LT owing to the massive immunosuppression involved. In such cases, antiviral therapy should be added in parallel until the appropriate revaccination time after LT (usually 3-6 mo) and until anti-HBs levels increase to ≥ 200 mIU/mL after revaccination.

Children with chronic HBV infections are rarely indicated for LT because they are usually asymptomatic in the stage of hepatitis B e-antigen (HBeAg)-positive chronic

infection or HBeAg-positive chronic hepatitis. Thus far, immunoprophylaxis data on recurrent HBV infections after LT are mainly based on adult data.

In summary, strategies to prevent HBV infection before, during and after LT mainly include active immunization. Super-accelerated and accelerated vaccines may be considered for timely protection prior to LT (to keep anti-HBs levels ≥ 1000 mIU/mL if possible). However, in children, anti-HBs levels should be regularly monitored, and revaccinations should be provided to maintain high anti-HBs levels (≥ 200 mIU/mL).

The future of HBV elimination after LT

Despite antiviral HBIG and active HBV immunization strategies, DNH has been reported in 0.9%-4.0% of both paediatric and adult LT patients[48,52,53]. Table 1 summarizes the risk factors for DNH. An escape mutation in the "a" determinant region within the hepatitis B surface antigen (HBsAg) that develops before LT, after HBV vaccination, or after HBIG administration post-LT should be considered[54]. In this situation, antiviral agents play a major role in preventing DNH, and long-term assessment for drug resistance should be considered. We recommend including pre-LT evaluations for HBV by serological, molecular and virological methods. Liver donors and allografts should be evaluated for covalently closed circular DNA (cccDNA) and HBV viral loads in cases of suspected occult infection with an escape mutant.

How to treat de novo hepatitis B infection in children after LT

Su *et al*[54] found that after DNH occurred in children post-LT, more than half (5/9) exhibit seroconversion after lamivudine therapy. However, one child carried a tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif mutation, and the authors switched antiviral agent from lamivudine to adefovir dipivoxil. To date, no consensus treatment for DNH has been reached[43,54-56]. Antiviral therapy for DNH might follow the guidelines for treating HBV infections in children (Table 2). In our unit, one patient with DNH was treated with interferon-α for 1 year without a response, even though this child exhibited HBsAg seroconversion after 6 mo of entecavir therapy. We revaccinated him against HBV after HBsAg clearance following entecavir therapy. This child received an HBV revaccination series (0, 1 and 6 mo) and maintained anti-HB levels of > 1000 mIU/mL without a rebooster at a 44-mo follow-up. Further study on the efficacy of antiviral therapy for DNH and other novel antiviral therapies with less drug resistance and high efficacy in children with DNH should be conducted to determine the best endpoints of HBsAg clearance and anti-HBs appearance. Tenofovir alafenamide (TAF) is a novel tenofovir product with improved properties for avoiding kidney and bone-related adverse events due to tenofovir disoproxil fumarate (TDF). Compared with TDF, TAF has non-inferior efficacy and a good safety profile[57]. Nevertheless, data for post-transplant adults and children receiving TAF are lacking. Only a small single-centre study of adult liver-transplant recipients found that TAF (25 mg/d) displayed high antiviral efficacy in preventing HBV recurrence without affecting immunosuppressive medications or graft functioning and had a good safety profile[58]. TAF is a promising antiviral therapy for adolescents diagnosed with DNH.

Other novel antiviral therapy

As mentioned, current HBV prophylaxis and therapies do not completely eradicate HBV infections in most cases, requiring lifelong medication. Thus, effective and finite HBV treatment remains an unmet medical need, and new therapeutic approaches and drugs are necessary to achieve a functional cure (mainly defined as a loss of when HBsAgs off therapy). Multiple novel drugs targeting different steps in the HBV life cycle are being developed. Antiviral and host-targeting agents are the two main drugs being studied. The major HBV-target-specific categories of antiviral drugs are hepatocyte-entry receptor inhibitors (*e.g.*, bulevirtide, formerly myrcludex B)[59,60], cccDNA inhibitors, nucleocapsid-assembly modulators (core protein allosteric modulators, *e.g.*, JNE-56136379)[61], post-transcriptional control inhibitors (RNA interference drugs, *e.g.*, ARC-520)[62], HBsAg-release inhibitors (nucleic-acid polymers, *e.g.*, REP 2139 and 2165)[63] and HBV DNA polymerase inhibitors. Therapies that target host immune responses include Toll-like receptor (TLR)-7 (*e.g.*, GS-9620, vesatolimod)[64], TLR-8 (GS-9688, selgantolimod)[65], and TLR-9 agonists, checkpoint inhibitors (anti-programmed death 1 and anti-programmed death-ligand 1)[66] and therapeutic vaccines[67]. These drugs are currently in phase I and II clinical trials that mainly include non-transplant adult patients and indicate a promising future for HBV eradication. No data are available on the efficacy of these new drugs

Table 1 Risk factors of *de novo* hepatitis B infection in children after liver transplantation

Risk factors
Positive anti-HBc donor[40]
Positive-intrahepatic HBV DNA[40]
Liver graft HBV DNA > 1000 copies[40]
Intraoperative fresh-frozen plasma transfusion > 400 mL[40]
Positive-anti-HBc recipients[40]
Pre-operative anti-HBs < 1000 mIU/mL[40,43,48]
Post-operative anti-HBs < 100-200 mIU/mL[48,53]
Hepatitis B surface mutation (within the "a" determinant region[54])

Anti-HBc: Hepatitis B core antibody; anti-HBs: Hepatitis B surface antibody.

Table 2 Antiviral agents for hepatitis B infection in children[44]

Medication	Licensing	Dose and duration	HBsAg loss (%)	Resistance (%)
IFN-α-2b	≥ 1 yr	6 million IU/m^2 three times weekly for 6 mo	1-2	0
Lamivudine	≥ 2 yr	3 mg/kg daily for ≥ 1 yr	0	19-64
Entecavir	≥ 2 yr	0.25-0.5 mg daily for ≥ 1 yr	0.52	0.7-1.2
Tenofovir dipovaxil fumarate	≥ 12 yr	300 mg daily for ≥ 1 yr	0.02	0
Adefovir	≥ 12 yr	10 mg daily for ≥ 1 yr	0	0.9-20

HBsAg: Hepatitis B surface antigen.

against HBV recurrence or *de novo* infection in children after LT. Further studies are needed to determine the impact of the new drugs on these patient groups.

Based on current knowledge of the human immunodeficiency virus (HIV) and hepatitis C virus (HCV), immunomodulators and combination treatments targeting several steps in HBV replication will likely be required to achieve a functional cure for HBV. Preclinical studies are applying this strategy in animal models[68], and clinical trials are investigating combinations of several antiviral drugs or immune boosters with antiviral agents. This new approach using combination therapies will need to be individualized, but many patients may be eligible.

In summary, strategies to eliminate HBV in paediatric liver transplant recipients include HBV immunization both pre- and post-LT. Early detection of HBV infections, especially of escape mutants, which lead to vaccine failure in recipients, and of cccDNA in the livers of positive anti-HBc donors, should be evaluated *via* molecular and viral genetic analysis in the liver tissues of both the donors and recipients. Patients with vaccine failure or DNH should promptly undergo antiviral therapy. Figure 2 shows the proposed strategies to eliminate HBV in children post-LT.

HEPATITIS C VIRUS

HCV infections are a global health problem, with an estimated 71 million people being chronically infected in 2016 and 400000 deaths annually worldwide[69]. Therefore, in 2016, the World Health Organization (WHO) set the goal of eliminating HCV by 2030. There has been significant progress towards this goal in screening policies, improving access to care, and reducing the costs of direct-acting antivirals (DAAs). Compared with adult patients, little attention has been paid to diagnosis, therapy, and prevention for children and adolescents. One reason is that prior to 2017, no DAAs were licensed for use in patients under 18 years old, and evidence was lacking to support paediatric management guidelines and policies. The majority of national HCV policies do not include explicit recommendations for HCV testing and treatment in children and

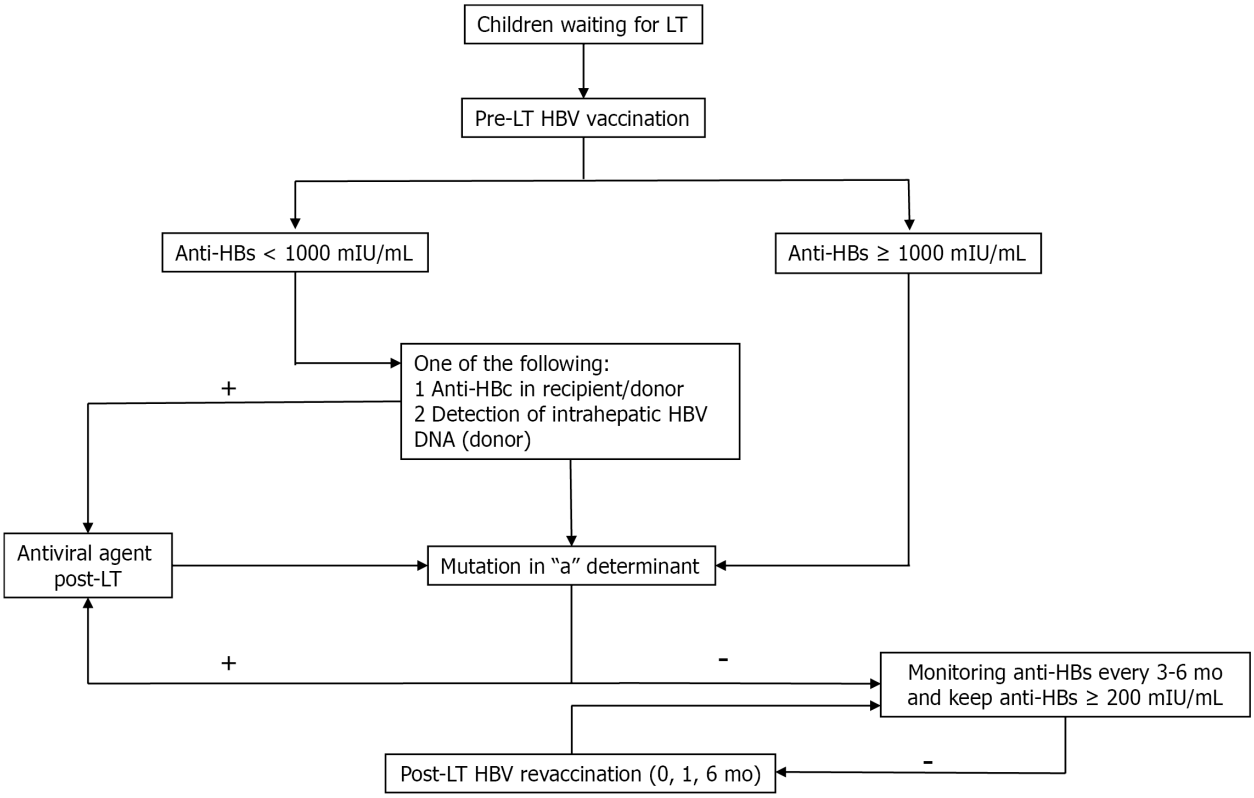


Figure 2 Proposed strategies to prevent *de novo* hepatitis B infection[41-48,52,53]. LT: Liver transplantation; HBV: Hepatitis B virus; anti-HBc: Hepatitis B core antibody; anti-HBs: Hepatitis B surface antibody.

adolescents[70]

Transmission route and natural history

In 2018, the global prevalence of HCV viraemia in populations under 18 years old was 0.13%, with an overall burden of 3.3 million cases[71]. The true HCV infection prevalence in paediatric populations is unknown due to a lack of universal screening strategies. Perinatal transmission is a major cause of recognized HCV infections in children, with transmission rates of 5% from HCV-infected mothers and 10% from HCV-HIV-coinfected mothers[72,73]. Moreover, the opioid epidemic is associated with an expanding ongoing risk of HCV transmission from mothers to children[74]. In the United States, nearly 29000 HCV-infected women gave birth annually from 2011-2014 [75]. Moreover, the transmission risk increases with higher maternal HCV viral loads, HIV coinfections, longer labour durations, amniocentesis or foetal-scalp monitoring, and prolonged membrane rupture[72,76-78]. Several studies from developed countries have reported increased injection drug use as a risk factor of HCV and HIV infections among adolescents[79,80]. Sexual transmission of HCV is also a major factor in men who have sex with men, including those infected with HIV or those who have received a pre-exposure prophylaxis for HIV[81,82].

After vertical HCV transmission, 25%-40% of patients spontaneously clear the infection within the first 4 years of life[83]. Approximately half of infants born with HCV will develop chronic disease that may lead to cirrhosis and hepatocellular carcinoma in late childhood[84]. The natural history of paediatric HCV differs from that of HCV acquired in adulthood. Host factors (*e.g.*, rs12979860 mutation in the *IL28B* gene[85], natural killer cell cytolytic functions[86]) and viral factors (*e.g.*, HCV genotype)[87] are associated with spontaneous clearance of HCV infections. Children with chronic HCV infections are mostly asymptomatic, with mild degrees of hepatitis and fibrosis during childhood and higher rates of spontaneous HCV clearance. Therefore, it is uncommon for children and adolescents to develop HCV-associated end-stage liver disease or hepatocellular carcinoma (HCC)[88]. Comorbidities, including haematological disease with iron overload, obesity, alcohol use, and concomitant viral infections (*e.g.*, HBV or HCV), are associated with accelerated liver fibrosis and cirrhosis development[89]. HCV-related extrahepatic manifestations are less common in paediatric patients than in adult patients[90]. In general, HCV

infections in children and adolescents are related to poor life quality and reduced cognitive functioning[84].

Diagnostic testing

Several current international guidelines recommend anti-HCV testing (with a confirmatory nucleic acid assay for a positive result) for all pregnant women, especially those in high-risk groups, including those with past or current injection drug use, incarceration history, unregulated tattoos/piercings, receipt of contaminated blood products, or exposure in HCV-endemic areas[91-93]. HCV RNA can be found in breast milk and colostrum, but breastfeeding does not increase HCV transmission rates except in HCV-HIV coinfected mothers[94]. All children born to HCV-infected mothers should be tested for HCV infection before 18 mo of age. Because anti-HCV antibodies passed from mothers can persist until 18 mo of age, HCV infection in children younger than 18 mo can be diagnosed by detecting HCV RNA. High-risk adolescents, including those who with histories of injection drug use and men who have sex with men, should be tested for HCV infection[95].

The asymptomatic nature of HCV infection and the high cost of diagnostic screening are the important barriers to detecting and treating HCV-infected patients [96]. Thus, a simple, cost-effective diagnostic method for routine HCV screening especially for low- to middle- income countries is needed. The core antigen of HCV (HCV Ag) is an alternative for screening and diagnosis. This test can be used as a supplemental marker after anti-HCV testing to reduce the requirement of further confirmatory HCV RNA assays[97]. Point-of-care tests of viraemia are related with improvement in access to testing [98].

Treatment DAAs in HCV infection before/after LT

Advancement of oral DAA therapies has resulted in a paradigm shift in treating HCV, with cure rates of > 90% and few adverse effects. DAAs with pan genotypic activity are recommended as preferred regimens for all treatment-naïve and treatment-experienced HCV patients, regardless of age, sex, stage of liver fibrosis, or HIV coinfection[93,99,100]. Conversely, pegylated-interferon-based regimens are no longer recommended. DAA treatment with an approved regimen is recommended for all children and adolescents ≥ 3 years old with HCV infection, regardless of disease severity[101,102]. Early antiviral treatment should be administered to reduce morbidity and mortality if extrahepatic manifestations occur (*e.g.*, glomerulonephritis and cryoglobulinemia).

Adolescents aged 12-17 years who are treatment-naïve or -experienced, without cirrhosis or with compensated cirrhosis (Child-Pugh A) should be treated according to the recommendations for adult patients. For pangenotypic HCV, two DAA regimens are recommended: sofosbuvir (400 mg)/velpatasvir (100 mg) once daily for 8-12 wk, achieving a 95% sustained virological response (SVR)-12 rate (97/102; 1 virological failure) with mild-to-moderate adverse events[103]; a fixed-dose combination of glecaprevir (300 mg)/pibrentasvir (120 mg) once daily for 8 wk, achieving a 100% SVR-12 rate with a good safety profile[104]. Although the clinical trial for glecaprevir /pibrentasvir included only adolescents with HCV genotypes 1-4, this drug was approved by the Food and Drug Administration (FDA) for adults with all genotypes. In 2019, the FDA approved treating genotype-specific HCV with sofosbuvir (400 mg)/ledipasvir (90 mg) for 12 wk in adolescents aged 12-17 years or weighing at least 35 kg with genotypes 1, 4, 5, or 6, without cirrhosis or with compensated cirrhosis[105, 106].

Children aged 3-11 years who are treatment-naïve or treatment-experienced, without cirrhosis or with compensated cirrhosis (Child-Pugh A) with any HCV genotypes should be treated with the FDA-approved regimens of a fixed-dose combination of sofosbuvir (200 mg)/velpatasvir (50 mg) for those aged > 6 years weighing ≥ 17 kg and sofosbuvir (150 mg)/velpatasvir (37.5 mg) for 12 wk[103] in those with weighing < 17 kg. One trial found that for children aged 3-11 years, the fixed-dose combination of glecaprevir (250 mg)/pibrentasvir (100 mg) for those weighing 30-44 kg, glecaprevir (200 mg)/pibrentasvir (80 mg) for those weighing 20-29 kg, and glecaprevir (150 mg)/pibrentasvir (60 mg) for those weighing 12-19 kg for 8-16 wk achieved a 96% SVR-12 rate, without drug-related severe adverse events[107]. However, this formulation is not yet FDA approved.

Overall, DAA-experienced children and adolescent patients with HCV are rare in clinical practice (Table 3). Because data for these populations are limited, DAA-experienced paediatric patients with HCV infections should be treated using the guidelines for adult patients.

Table 3 Recommended direct-acting antiviral regimens for children who are naïve to or experienced with direct-acting antiviral therapy [101,102]

Age	Genotype	No cirrhosis/ cirrhosis	Recommended regimens of DAAs	Duration (wk)
12-17 yr	Pan-genotypes	No cirrhosis	Sofosbuvir 400 mg/ velpatasvir 100 mg	12
		Compensated cirrhosis (Child-Pugh A)	Glecaprevir 300 mg/pibrentasvir 120 mg	8-12
12-17 yr or BW ≥ 35 kg	1, 4, 5, 6	No cirrhosis	Sofosbuvir 400 mg/ledipasvir 90 mg	12
		Compensated cirrhosis (Child-Pugh A)	Sofosbuvir 200 mg/velpatasvir 50 mg (BW ≥ 17 kg)	
3-11 yr	Pan-genotypes	No cirrhosis	Sofosbuvir 150 mg/velpatasvir 37.5 mg (BW < 17 kg)	12
		Compensated cirrhosis (Child-Pugh A)	Glecaprevir 250 mg/pibrentasvir 100 mg (BW 30-44 kg); Glecaprevir 200 mg/pibrentasvir 80 mg (BW 20-29 kg); Glecaprevir 150 mg/pibrentasvir 60 mg (BW 12-19 kg)	12; 8-16; 8-16; 8-16;

BW: Body weight.

LT in paediatric patients in the DAA era

Children and adolescents with chronic HCV infections rarely require LT for complications from liver cirrhosis or HCC; recurrent HCV after LT is also clinically rare. In a retrospective study of the United Network of Organ Sharing database, Gupta *et al*[108] found that 120 paediatric patients received transplants for chronic HCV infections in 1994-2010. One-year and 3-year survival rates were 97% and 89%, respectively, in patients with post-paediatric end-stage liver diseases. Pre-LT recipient factors, good surgical technique, and effective treatment for HCV infections are associated with good prognostic outcomes in paediatric patients after LT[108]. Patients who achieve an SVR have less mortality than do those without SVR after treatment. Treatment has better effects on disease outcomes if it is started before cirrhosis[109]. To prevent long-term liver disease and HCV spread, antiviral therapy should be available in childhood.

LT for HCV-related diseases has decreased in the era of DAA treatment. Treating patients before LT reduces the chance of graft dysfunction after LT and may stabilize or improve liver function. SVR before LT may lead to the delisting of some patients [110]. Patients with decompensated (Child-Pugh B or C) cirrhosis who have model for end-stage liver disease (MELD) scores of < 20 without HCC and are awaiting LT should be treated with DAAs before LT. The recommended regimen is sofosbuvir (400 mg)/velpatasvir (100 mg) plus weight-based ribavirin 1000-1200 mg/day for 12 wk or sofosbuvir (400 mg)/velpatasvir (100 mg) for 24 wk in those with contraindications for ribavirin. Patients with MELD scores > 20 should undergo transplantation first and treated for HCV infection after LT if the waiting time is < 6 mo[111,112].

Future treatment

Vaccines: Despite the high curative rate of HCV infections by DAAs, high-risk populations remain at risk of reinfection, even after successful treatment. Preventing new HCV infections is vital and may result in the WHO's 2030 global elimination goal. A prophylactic HCV vaccine might also help to achieve this goal by preventing transmission. Nevertheless, no vaccine for preventing HCV infections has been approved to date.

A recent phase 1-2, randomized, double-blind, placebo-controlled trial by Page *et al* [113] enrolled adults aged 18-45 years who had injected drugs within 90 d. These adults received either an intramuscular injection of a recombinant chimpanzee adenovirus type-3 vector-priming vaccination (ChAd3-NSmut vaccine) on day 0 and a recombinant modified vaccine (Ankara, MVA-NSmut vaccine) booster on day 56 (vaccine group) or a saline placebo on days 0 and 56. Despite inducing HCV-specific T-cell responses and lowering peak HCV RNA levels, the vaccine failed to prevent chronic HCV infection compared with placebo[113]. The innate variability of HCV enveloped proteins and the limited knowledge of HCV protein structures are barriers to developing an HCV vaccine. Future work should determine the optimal HCV epitopes to target vaccine development.

HEPATITIS E VIRUS

Hepatitis E virus (HEV) was first discovered in the 1980s and normally manifests as an acute self-limited condition[114], though chronic HEV infection courses were recognized in 2008 in organ-transplant recipients[115]. HEV infection seroprevalence varies from 0.3%-75.6% depending on the area and diagnostic method[116-121]. HEV is transmitted mainly *via* the faecal-oral route, but mother-to-child[122], liver graft-to-recipient and plasma-derived-product transmission[123,124] have been reported. One study reported HEV transmission *via* liver graft[125], and several cases of HEV infections transmitted by blood transfusion have been reported. These findings have led to universal HEV-RNA testing in blood donors in many countries[123,124,126,127]. To detect HEV infection in immunocompetent children, primary testing with anti-HEV IgG and IgM is reasonable, and HEV RNA in stool and serum samples should be assessed in highly suspected cases that yielded negative results by serological methods[128]. However, serum HEV RNA analysis is preferable in immunocompromised patients, as they cannot mount an antibody response[129]. HEV infection has clinical impacts in immunocompromised hosts, especially in those needing organ transplantation. Moreover, HEV infections can be asymptomatic pre-existing chronic liver diseases or solid organ transplantation[130-133], or liver-associated morbidity due to progressive fibrosis and cirrhosis may be present[134]. Additionally, these conditions increase the risk of graft rejection[132]. In 2014, the European Association for the Study of the Liver proposed a well-organized stepwise plan for managing HEV infections in both adults and children after organ transplantation[128]. Once HEV infection is detected in children after LT, immunosuppression should be reduced when possible, and these children should be followed up within 3 mo. HEV-RNA clearance may occur in one-third of these patients. If chronic HEV infection persists, antiviral therapy with ribavirin (15 mg/kg/d) should be administered for at least 3 mo[135], and HEV clearance should be monitored monthly *via* PCR. Three promising recombinant vaccines against HEV with high efficacy have been developed[136] and can maintain seroprotection for > 4.5 years[137,138]. Many studies and case reports of HEV-infected children after LT have resulted from this increased awareness. Table 4 shows the results of previous studies[130-133,139,140] and HEV infection data for children after LT in our centre (unpublished data).

Strategies to eliminate HEV infection include prevention by implementing hygienic measures and thoroughly cooking food, screening plasma-derived products from immunocompromised patients and, developing an HEV vaccine. Early detection and effective treatment with antiviral agents in infected patients are also crucial.

Future: How to eliminate HEV after LT

Diagnostic testing for HEV infection: As chronic HEV infections in children after LT lead to progressive hepatitis and liver fibrosis, suspected cases should be tested. As serologic testing is insufficient to detect HEV infections in immunosuppressed patients, HEV infections should be diagnosed based on HEV-RNA detection in specimens. Protzer *et al*[141] reported molecular detection of HEV in liver-biopsied tissues from four liver-transplanted patients, whereas serology only detected two (Mikrogen assay). Prost *et al*[142] compared HEV-RNA detection in clinical liver-biopsy tissues between *in situ* testing and qPCR from paraffin-embedded liver tissues and found that qPCR was more effective. Additionally, Ankavay *et al*[143] found that detecting the open reading frame 2 (ORF2) protein of HEV *via* immunohistochemistry of liver tissues can be used as a rapid histopathological method to diagnose HEV infections. The sensitivity and specificity of this technique were the same as those of tissue PCR for HEV RNA. The ORF2 clone 1E6 antibody yielded the highest diagnostic accuracy and was more sensitive for HEV serotypes 1 and 3 in the livers of both immunocompromised and immunocompetent patients[143]. Detecting HEV in liver tissues may be more reliable and may correlate directly with liver inflammation and damage in the immunocompromised. Regardless, a limitation of ORF2 clone 1E6 staining is that it is less sensitive for HEV genotypes 2 and 4. A cost-effective method of detecting HEV infection with high efficacy is still needed. Table 5 summarizes the HEV detection methods and their diagnostic value[144,145].

Antiviral therapy for HEV infection: In addition to ribavirin, other medications used in HEV-infected adults include pegylated interferon-α and add-on effects of sofosbuvir with ribavirin[128,146]. Recent data show that interferon λ1-3 inhibits HEV replication in an *in vitro* culture system and may be effective for treating HEV infections[147]. Another proposed medication is zinc salt. In a human hepatoma cell study, zinc salt dose-dependently inhibited replication of HEV genotypes 1 and 3[148]. In fact, zinc

Table 4 Studies of children infected with hepatitis E virus after liver transplantation

Ref.	Year	Country	Participants	Seroprevalence of HEV infection	Methods		Comments
					HEV IgM/G	HEV RNA	
1[130]	2012	Canada	Gr 1; N: 66 with normal LFT, aged 13.7 yr (1.8-25.5); Gr 2; N: 14 with transaminitis, aged 17.4 yr (5.9-19.8)	Gr 1: 10/66 (15%) with IgG +, none had IgM, HEV RNA +; Gr 2: 12/14 (86%) with IgG+; 9/12 (75%) with IgM+; 1/12 (0.8%) with HEV RNA +	Feldan Bio Inc, Saint-Augustin	Serum nested RT-qPCR	All in Gr 2 showed a trend toward chronic hepatitis and fibrosis; An 8-yr-old girl had chronic HEV infection (genotype 3) for > 10 yr and developed cirrhosis
2[131]	2012	Germany	N: 41 liver-transplanted children, aged 8.8 ± 4.2 yr	2/41 (4.9%) IgG +0/41 stool HEV RNA +	Mikrogen	Stool RT-qPCR	No case with chronic HEV infection
3[132]	2013	Germany	N: 22 liver-transplanted children, aged 6.7 yr (1.4-17.2)	1/22 (0.45%) IgG + by Wantai assay and HEV RNA + in serum	Wantai assay	Serum or stool PCR	10-year-old boy with HEV infection that had persistent transaminitis after 2-mo immunosuppressive reduction. Ribavirin 15 mg/kg/d was started for 6 mo. Normal LFT and undetectable serum and stool HEV RNA at day 42 of treatment.
4[139]	2014	Brazil	One liver-transplanted child: case report	HEV IgG/IgM and HEV RNA in serum and liver tissue at 6-10 yr after liver transplantation	Mikrogen	Liver and serum RT-PCR	A 4-yr-old girl with transaminitis from ACR at 6 yr after LT, had transaminitis off and on and HEV IgG/IgM and HEV RNA was detected 9-10 yr after LT. Chronic HEV infection was successful treatment with ribavirin for 10 mo.
5[133]	2015	France	84 liver-transplanted children, aged 12.3 yr	8/84 (8.3%) HEV IgG+	Wantai assay	Ceeram Tools® kit for HEV-RNA detection	None had HEV IgM/RNA +; No case of chronic infection
6[140]	2020	France	80 liver-transplanted children, aged 3.5 ± 4 yr	6/80 (8%) with HEV IgG+	Wantai assay	Ceeram Tools® kit for HEV-RNA detection	None had HEV IgM/RNA +; No case of chronic infection; 4/6 had undetectable HEV IgG after follow-up (3-42 mo)
7	2021	Thailand	30 liver-transplanted children with transaminitis, aged 1.2-17.6 yr	14/30 (45.2%) with HEV IgG+, 4 (13%) with HEV IgM+ and one case with HEV RNA in stool	Euroimmun kit	Stool PCR	All of them had persistence of HEV IgM from 5 to 44 mo and transaminitis from 4 to 30 mo before HEV testing. The previous treatment included graft rejection, *de novo* autoimmune hepatitis and CMV viremia.

Ref: Reference; Gr: Group; RT-qPCR: Real-time polymerase chain reaction; HEV: Hepatitis E virus; ACR: Acute cellular rejection; MP: MP Biomedicals, formerly Genelabs Diagnostics, Singapore; Wantai assay: Wantai Biologic Pharmacy Enterprise, Beijing, China; LT: Liver transplantation; CMV: Cytomegalovirus.

Table 5 Diagnostic tests for hepatitis E infection[144,145]

Detection	Technique	Specimen
Virus or its components (direct method)	**HEV nucleic acid:** (1) RT-PCR; (2) Realtime RT-PCR; and (3) Loop-mediated isothermal amplification assay. **HEV RNA:** (1) *In situ* hybridization; (2) HEV viral protein (antigen); (3) EIA; and (4) IHC.	Serum, stool, bile, liver tissue
Host immune response (indirect method)	**Specific anti-HEV antibodies (IgM and IgG)** (sensitivity 72%-98% and specificity 78%-96%): (1) Indirect EIA; (2) Immunochromatographic assays; (3) Double-antigen sandwich-based EIAs; (4) μ capture EIAs for IgM anti-HEV; (5) Specific cellular immune response; and (6) ELISpot assays.	Serum, peripheral blood mononuclear cells

HEV: Hepatitis E virus; RT-PCR: Reverse transcription polymerase chain reaction; ELISpot: Enzyme-linked immune absorbent spot; EIA: Electroimmunoassay; IHC: Immunohistochemistry.

can directly decrease HEV replication by suppressing viral translation and processing of nonstructural proteins encoded by ORF1 and by inhibiting IFN-λ3 from binding to its receptor[149,150]. Moreover, zinc has an indirect effect by modulating host immune

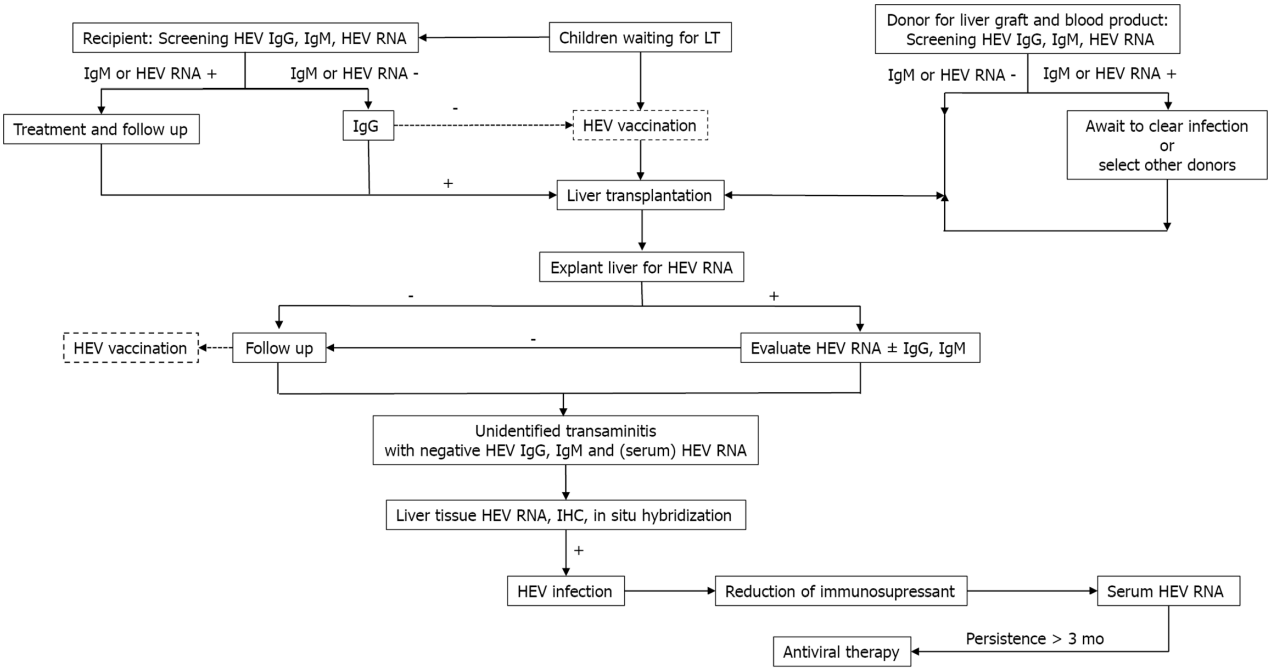


Figure 3 Proposed strategies to eliminate hepatitis E virus infection in children after liver transplants[127,128,130,135,141,142]. LT: Liver transplantation; HEV: Hepatitis E virus; IHC: Immunohistochemistry.

responses and is a cofactor in host cellular processes[150]. Hence, zinc is a promising drug for HEV therapy without serious adverse effects. Clinical and basic research are needed regarding the therapeutic benefits of zinc in HEV infections.

Prevention with an HEV vaccine: Since 2001, several vaccines based on virus-like particles have been developed[151], and there have been clinical trials on three vaccine candidates[136,137,152]. One is licensed in China, with 100% efficacy over 12 mo after 3 injections[137]. Moreover, the efficacy remained high at 86.8% after a 4.5-year follow-up[138]. However, these three vaccines mainly protect against genotypes 1 and 4 but cannot protect against genotype 3, which is the main genotype causing chronic HEV infections in patients after LT[153]. In 2019, an HEV vaccine was initiated and is progressing in clinical trials in the United States[153]. In general, an HEV vaccine will be a powerful weapon in public health for protecting against HEV infections (Figure 3).

CONCLUSION

To eliminate viral hepatitis in paediatric liver-transplant recipients, multiple strategies must be integrated into clinical practice. Similar to the prevention of HAV infections, immunization is the mainstay of prevention against HBV infection in children with liver transplants. Regular monitoring of humoral immunity for HBV and HAV and revaccination programmes in cases with immunity loss are necessary. Antiviral therapy plays a major role in HBV and HCV infections. For HEV infection, molecular techniques for early detection in children with liver transplant with unidentified causes of hepatitis should be developed to guide proper management of HEV infection.

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REVIEW

Non-alcoholic fatty liver disease and hepatocellular carcinoma: Clinical challenges of an intriguing link

Lampros Chrysavgis, Ilias Giannakodimos, Panagiota Diamantopoulou, Evangelos Cholongitas

ORCID number: Lampros Chrysavgis 0000-0003-2632-0742; Ilias Giannakodimos 0000-0003-2267-8464; Panagiota Diamantopoulou 0000-0001-9403-7696; Evangelos Cholongitas 0000-0002-3645-582X.

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Lampros Chrysavgis, Department of Experimental Physiology, National and Kapodistrian University of Athens, Athens 11527, Greece

Ilias Giannakodimos, Panagiota Diamantopoulou, Evangelos Cholongitas, First Department of Internal Medicine, "Laiko" General Hospital of Athens, National and Kapodistrian University of Athens, Athens 11527, Greece

Corresponding author: Evangelos Cholongitas, MD, PhD, Associate Professor, First Department of Internal Medicine, "Laiko" General Hospital of Athens, National and Kapodistrian University of Athens, Agiou Thoma 17, Athens 11527, Greece.
cholongitas@yahoo.gr

Abstract

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common liver disorder worldwide mainly attributed to the epidemic spread of obesity and type 2 diabetes mellitus. Although it is considered a benign disease, NAFLD can progress to non-alcoholic steatohepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Most data regarding the epidemiology of NAFLD-related HCC are derived from cohort and population studies and show that its incidence is increasing as well as it is likely to emerge as the leading indication for liver transplantation, especially in the Western World. Although cirrhosis constitutes the main risk factor for HCC development, in patients with NAFLD, HCC can arise in the absence of cirrhosis, indicating specific carcinogenic molecular pathways. Since NAFLD as an underlying liver disease for HCC is often underdiagnosed due to lack of sufficient surveillance in this population, NAFLD-HCC patients are at advanced HCC stage at the time of diagnosis making the management of those patients clinically challenging and affecting their prognostic outcomes. In this current review, we summarize the latest literature on the epidemiology, other than liver cirrhosis-pathogenesis, risk factors and prognosis of NAFLD-HCC patients. Finally, we emphasize the prevention of the development of NAFLD-associated HCC and we provide some insight into the open questions and issues regarding the appropriate surveillance policies for those patients.

Key Words: Non-alcoholic fatty liver disease; Hepatocellular carcinoma; Epidemiology; Risk factors; Surveillance; Risk stratification

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is projected to emerge as the leading cause of hepatocellular carcinoma (HCC) worldwide. Demographic factors, genetic predisposition and behavioral parameters have been identified as independent risk factors for NAFLD-related HCC, which can arise even in the absence of cirrhosis. Currently, the most challenging issue for the scientific community worldwide is the identification of the pre-cirrhotic NAFLD patients who have increased risk for HCC. Noteworthy, the central concept for the surveillance policies in the near future should be the identification, *via* an individual, risk-assessment based precision screening of high-risk NAFLD patients, cirrhotic or not.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as the presence of triglycerides ≥ 5% into the hepatic tissue (*i.e.* steatosis), in the absence of excessive alcohol consumption and other competing liver disorders such as chronic viral hepatitis or administration of steatogenic drugs[1]. The disease may progress to non-alcoholic steatohepatitis (NASH) which is characterized by steatosis and liver inflammation, with or without fibrosis. NAFLD is considered as the global epidemic of the 21st century in the field of liver diseases and is strongly associated with the increased prevalence of obesity[1,2]. In 2016, the World Health Organization estimated the number of overweight or obese adults to be more than 1.9 billion worldwide. NAFLD is also correlated with other metabolic comorbidities, besides obesity, namely type 2 diabetes mellitus (T2DM), hyperlipidemia, arterial hypertension and it is considered the hepatic manifestation of the metabolic syndrome (MetS)[3]. Concerning epidemiology of NAFLD, a recent large meta-analysis, including 45 studies reported an estimated global prevalence of NAFLD as high as 25.24% with highest prevalence in the South America (30.45%) and Middle East (31.8%) and lowest in Africa (13.5%)[4]. Of note, during the past decade, a consistent rise of NAFLD prevalence was observed, increasing from 15% in 2005 to 25% in 2010[4].

Concerning the advanced form of the disease, the pooled NASH prevalence among NAFLD patients with an indication for liver biopsy was 63.45% for Asian region, 69.25% for Europe and 60.64% for North America[4]. Oppositely, NASH prevalence among NAFLD patients without an indication for biopsy was 6.67% for Asia and 29.85% for North America, while no corresponding data were available for European territory[4]. Moreover, approximately 41% of NASH patients experienced fibrosis progression with an average annual progression rate of 0.09%[4]. A smaller, but significant proportion of NAFLD and mainly NASH patients will ultimately develop cirrhosis or even hepatocellular carcinoma (HCC), thus facing life-threatening liver-associated complications[5]. To our point of view, since prevalence of NAFLD is increasing rapidly globally, HCC in patients with NAFLD will become a major public health issue and will emerge as a leading cause for liver transplantation (LT) in the near future. The vast underestimation of the true burden of NAFLD, especially in the developing countries, leads to reduced and delayed access of patients to specialized medical centers for appropriate surveillance and treatment of HCC and its complications, since early diagnosis constitutes a fundamental factor for effective therapy.

In this current review, we discuss the latest data concerning epidemiology, risk factors and prognosis of NAFLD-related HCC as well as we emphasize the prevention, and the appropriate surveillance polices which shall be conducted to improve patients' attentiveness and care.

LITERATURE SEARCH

We reviewed the current literature from the inception of this current review until March 2021. For our scope, we used "PubMed" database and we included only studies written in English. We used the following search terms: "Non-alcoholic fatty liver disease-related hepatocellular carcinoma", "NAFLD-related HCC", "NAFLD-HCC", "Non-alcoholic steatohepatitis-related hepatocellular carcinoma", "NASH-related HCC", "NASH-HCC" and we retrieved the results of our search for the epidemiology, pathogenesis, risk factors, prognosis/outcomes, surveillance, and prevention of NAFLD/NASH-related HCC. Also, the references of the research articles were scrutinized for relevant studies.

EPIDEMIOLOGY OF NAFLD-RELATED HCC

HCC, as an entity, is the fifth most frequently diagnosed cancer and the second leading etiology of cancer-related mortality worldwide[6]. It is the predominant histological type of primary liver cancer accounting for 70%-85% of all liver cancer cases[6] and is estimated to be the fastest growing cause of cancer-related mortality among United States male population[7]. Although most HCC cases occur in the setting of chronic viral hepatitis or alcoholic (ALD) cirrhosis, a significant proportion of patients with NAFLD/NASH may develop HCC. According to the aforementioned recent meta-analysis, the annual incident rate of HCC in NASH patients was 5.29 *per* 1000 person-years (PY), whereas for NAFLD patients that percentage dropped to 0.44 *per* 1000 PY[4]. Noteworthy, another meta-analysis which included only studies of Asian populations reported that the incidence rate of HCC was 1.8 cases *per* 1000 PY on NAFLD patients, while the corresponding data for NASH patients were unavailable due to the design of that meta-analysis[8]. The prevalence of NAFLD-related HCC is rising worldwide, and data derived from studies conducted in the past decade estimated that 4%-22% of all HCC were attributed to NAFLD in Western countries with the corresponding percentage to be 1%-2% in Asian region, where viral hepatitis remains endemic[9]. However, these studies underestimate the true NAFLD-related HCC prevalence, as they ignore the impact of "cryptogenic" cirrhosis. It is widely suspected that half of the cryptogenic cirrhosis-related HCC, which accounts for 15% to 30% of all HCC cases, arise from NAFLD[10].

Regarding the prevalence of HCC among NASH patients (cirrhotic or not), as compared to other liver diseases, a recent meta-analysis demonstrated that it was 22.5% for all NASH and 13.6% among all other non-NASH patients[11]. More recently, a large health-care database study in the United States identified NAFLD or NASH as the most predominant underlying risk factor for HCC, being present in 59% of cases [12]. In addition, NAFLD accounted for 34.8% of HCC events in England, while a year-by-year increase of HCC attributed to NAFLD between 2000 and 2010 was identified [12,13]. In a recent analysis of the Scientific Registry of Transplant Recipients including data from 158347 candidates for LT in the United States between 2002 and 2016, the proportion of NAFLD/NASH in HCC was increased 7.7-fold (from 2.1% to 16.2%), while the corresponding proportion for other-etiologies HCC remained relatively stable[14]. Of note, during this period the prevalence of HCC in LT candidates with NASH increased 11.8-fold[14]. Consistent with that, during a 20-year period, among a French cohort of histologically confirmed HCC patients who underwent surgical resection, the prevalence of NAFLD-related HCC increased from 2.6% in the period of 1995-1999 to 19.5% in 2010-2014, while the corresponding hepatitis C virus (HCV)-related fraction was decreased from 43.5% to 19.5%[15]. Along this line, Hester *et al*[16] in their recent cross-sectional study including 13648 HCC patients, identified NAFLD as the predominant cause of HCC in both inpatient and outpatient population, accounting for 32.07% and 20.22% of all cases respectively, followed by HCV infection [16].

Of note, a major cause for concern is the incidence of HCC among T2DM patients considering both the high prevalence of T2DM globally and the fact that > 70% of T2DM patients have NAFLD[17]. A large observational study revealed that HCC was the most incident malignancy among 457473 T2DM patients (Hazard Ratio: 3.31), while a large population-control study further confirmed that T2DM was independently associated with 2-3-fold increase of HCC risk regardless of other well-established risk factors for HCC[18]. Interestingly, Dyson *et al*[13] showed that the prevalence of T2DM or obesity among HCC patients was growing during a decade of follow-up (2000-2010) while, intriguingly, in one third of all HCC patients referred to

this tertiary care centre, metabolic dysregulation was the only identified risk factor for HCC[13]. Of importance, numerous meta-analyses have also demonstrated similar findings[19-21]. We should emphasize that throughout the above-mentioned meta-analyses, the association between T2DM and HCC was robust across different population groups, geographic areas, and a plethora of control groups while it remained significant even after adjusting for demographic and laboratory parameters.

Of cardinal importance, a distinctive feature of NAFLD/NASH, compared to other liver diseases such as HCV or ALD, is the development of HCC even in the absence of cirrhosis[22]. In a retrospective study of 1500 patients with HCC, the incidence of HCC development without cirrhosis was higher in NAFLD patients, since 34.6% of NAFLD-HCC patients were non-cirrhotic, while only 8.9% in HCV, 7.7% in hepatitis B virus (HBV) and 11.1% in ALD groups had no evidence of cirrhosis[23]. Consistently, two large independent Western studies showed that 54% and 46.2% of NAFLD and NASH-related HCCs respectively, arose in a non-cirrhotic background[12,24], while the corresponding proportion was similar (49%) in a cross-sectional multicenter Japanese study[25]. Additionally, a recent meta-analysis confirmed those findings since in non-cirrhotic NASH subjects the pooled prevalence of HCC was 38% compared to 14.2% in non-cirrhotic non-NASH[11] suggesting that the former had significantly increased odds for developing HCC[11]. Thus, NAFLD seems to be the second cause, together with HBV, where HCC can develop in non-cirrhotic liver. Studies of the last decade concerning the epidemiology of NAFLD-related HCC are summarized in Table 1[13,15,16,23,26-45], while studies including cohorts of NAFLD patients who prospectively developed HCC are summarized in Table 2[24,25,46-52].

PATHOGENETIC PATHWAYS

Like other malignancies, NAFLD-related HCC development is a chronic process with gradual transition from the state of NAFLD to the state of cirrhosis and HCC onset. In the setting of liver cirrhosis, the pathophysiological mechanisms of HCC arising have been well-studied. Repeated cycles of hepatocyte death and subsequent liver regeneration and tissue restoring along with cellular proliferation and constant cell growth lead to tumor development[53]. Besides hepatic cirrhosis, in this current review we shed light to several other etiologies and mechanisms that have been specifically implicated in the development of NAFLD-related HCC, since a lot of non-cirrhotic related HCC are associated with NAFLD[36]. The potential pathogenetic molecular pathways implicated in the NAFLD-related HCC development are illustrated in Figure 1 (Created with BioRender https://biorender.com/). Herein, we highlighted the major and well-established pathogenetic mechanisms whereas the remaining and relatively recently proposed ones are in detail described in the Supplementary material.

NAFLD is closely associated with insulin resistance and subsequently increased levels of insulin and insulin-like growth factor-1 (IGF-1)[54]. Binding of these two molecules to insulin receptor and insulin-like growth factor-1 receptor (IGF1R) respectively, results in activation of PI3K/AKT and Mitogen-activated protein kinase (MAPK) molecular pathways[55]. Regarding the first, exerts its action by signaling on cyclin D1, Mdm2/p53 and mTOR and leads to inhibition of apoptosis, induction of cell proliferation and excessive cell growth respectively, while the activation of MAPK mediates the transcription of proto-oncogenes c-fos and c-jun, further affecting cell growth[56]. Moreover, MAPK pathway facilitates the activation of Wnt/β-catenin signaling cascade, which leads to liver fibrosis and promotion of hepatocarcinogenesis [57]. Moreover, insulin resistance and energy imbalance drive to excessive liver lipid accumulation, metabolic reprogramming and production of free fatty acids (FFAs) [58]. Increased mitochondrial oxidation of these FFAs induces the formation of reactive oxygen species (ROS) leading to insufficient mitochondrial respiratory chain activity as well as triggering apoptotic death pathways, such as receptor interacting protein 1 (RIP1) and RIP3- activated Jun-(N)-terminal kinase (JNK),which in turn facilitate liver inflammation and fibrosis[59,60]. In addition, the increased FFA oxidation is associated with augmented levels of endoplasmic reticulum (ER) and oxidative stress in hepatocytes. The latter promotes increased calcium release from ER that leads to mitochondrial permeabilization, disrupted ER function, liver cell injury and tumorigenesis in NASH[61]. Moreover, the crosstalk between oxidative or ER stress and ROS overproduction aggravates the progression of liver disease into NASH and HCC as the aforementioned mitochondrial dysfunction leads to further overproduction of ROS, which facilitate the activation of proapoptotic paths, mediating by

Table 1 Epidemiology of non-alcoholic fatty liver disease-related hepatocellular carcinoma based on studies published in the last decade (2011-2020)

Ref.	Year/Country	Study design	HCC patients, *n*	HCC caused by NAFLD, *n* (%)	Non-cirrhotics among HCC-related NAFLD, *n* (%)	Fibrosis stage of NAFLD-HCC patients, *n* (%)	Tumor size
Yang *et al* [26]	2011/United States	Retrospective	460	61 (13.27)	NA	NA	NA
Schütte *et al* [27]	2014/Germany	Retrospective	664	43 (6.5)	6 (13.95)	NA	NA
Chun *et al* [28]	2014/United States	Retrospective	27	13 (48.1)	NA	NA	NA
Edenvik *et al*[29]	2015/Sweden	Retrospective	616	69 (11.2)	15 (21.7)	NA	NA
Younossi *et al*[30]	2015/United States	Retrospective	4979	701 (14.1)	NA	NA	NA
Weinmann *et al*[31]	2015/Germany	Retrospective	1119	45 (4)	10 (22.2)	NA	Trend towards ↑ tumor size in NASH-HCC (6 cm) *vs* non-NASH-HCC (4.8 cm) (P = 0.18)
Mittal *et al* [23]	2016/United States	Retrospective	1500	107 (8)	37 (34.6)	NA	NA
Wong *et al* [32]	2017/United States	Retrospective	17.664	5898 (33.4)	3326 (56.4%)	NA	↑ proportion of tumors > 5 cm in NAFLD-HCC *vs* non-NAFLD-HCC (P < 0.001)
Huang *et al* [33]	2017/Australia	Prospective	270	38 (14)	9 (23.7)	NA	NA
Koh *et al*[34]	2019/Singapore	Prospective	996	152 (15.3)	100 (65.8)	F0 = 78 (51.7); F1 = 10 (6.6); F2 = 45 (29.8); F3 = 9 (6); F4 = 9 (6)	↓ tumor size in NAFLD-HCC (0.7 cm) *vs* non-NAFLD-HCC (4 cm) (P < 0.001)
Hassan and Gane[35]	2019/New Zealand	Retrospective	1985	159 (5.1) (Undefined cirrhosis stage in 57)	25 (24.5) (based on well-defined stage patients)	F0 = 2 (8); F1 = 3 (14); F2 = 1 (3); F3/4 = 19 (75.5)	NA
Gawrieh *et al*[36]	2019/United States	Retrospective	5144	767 (14.9)	159 (26.3)	NA	NA
Hester *et al* [16]	2020/United States	Retrospective	12471	3019	1565	NA	NA
Hong *et al* [37]	2018/Australia	Prospective	272	39 (14.3)	NA	NA	NA
Jamwal *et al* [38]	2020/India	Prospective	56	20 (35.7)	20 (100)	NA	NA
Pais *et al*[15]	2017/France	Retrospective	323	39 (12.1)	30 (76.9)	F0 = 16 (40); F1 = 9 (23); F2 = 0 (0); F3 = 5 (14); F4 = 9 (23)	↑ tumor size in NAFLD-HCC (8.7 cm) *vs* non-NAFLD-HCC (6.2 cm) (P = 0.002)
Dyson *et al* [13]	2013/United Kingdom	Prospective	632	136 (21.5)	31 (22.8)	NA	NA
Phipps *et al* [39]	2020/United States	Retrospective	5327	790 (14.8)	NA	NA	NA
Bengtsson *et al*[40]	2019/Sweden	Retrospective	1562	225 (14.4)	83 (36.9)	F0 = 1; F1 = 13; F2 = 16; F3 = 5 (Undefined fibrosis stage in 48 patients)	NSD in tumor size between NAFLD-HCC *vs* non-NAFLD-HCC; ↑ tumor size in non-cirrhotic *vs* cirrhotic NAFLD-HCC (P = 0.001)
Tokushige *et al*[41]	2013/Japan	Retrospective	14.530	292 (2)	111 (38)	NA	NA
Reddy *et al* [42]	2012/United States	Retrospective	303	52 (NASH) (17.2)	14 (26.9)	NA	NSD in tumor size between NAFLD-HCC (3.2 cm) *vs*

							non-NAFLD-HCC (3 cm)
Phan *et al* [43]	2019/United States	Retrospective	545	28 (5.1)	3 (10.7)	NA	NA
Van Meer *et al*[44]	2016/Netherlands	Retrospective	1221	181 (14.8)	67 (28)	NA	↑ tumor size in NAFLD-HCC (6 cm) *vs* HCV-HCC (3 cm) ($P < 0.001$)
Yang *et al* [45]	2017/United States	Retrospective	93	10 (11)	3 (27.3)	NA	NA

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus; NSD: No significant difference; NA: Not applicable.

caspases 9 and 3[62,63].

Furthermore, the products from lipid peroxidation and the elevated levels of ROS provoke the release of several pro-inflammatory and inflammatory substances such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) as well as affects adipokines' secretion, namely leptin and adiponectin[64]. Increased expression of IL-6 activates the oncogenic pathway of signal transducer and activator of transcription 3 (STAT-3) which mediates cell proliferation, inhibits apoptosis and contributes to HCC development, while augmented TNF-α levels mediate the activation of pro-oncogenic paths namely nuclear factor kB (NF-kB) *via* JNK and phosphorylation of inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKKβ)[65]. Consistently, leptin, as a profibrotic and proangiogenic factor exerts its action by both stimulating an intracellular signaling cascade of the inflammatory molecules namely TNF-α and IL-6 and by activating the previously mentioned JAK2/STAT-3, MAPK and PI3K pathways upon its binding to its receptor in HCC cells[66]. Noteworthy, although adiponectin is a strong anti-inflammatory mediator which modulates apoptosis under normotropic conditions, the exacerbated insulin resistance suppresses its action while ROS-induced overproduction of leptin, as an antagonistic hormone on the field of hepatic fibrogenesis with adiponectin, further inhibits its production and thus intensifies HCC development[67,68].

As the activation of the immune system considered as a prerequisite for NAFLD progression to NASH, the involvement of immunological pathways in the NAFLD-related HCC is of much interest. Ma *et al*[69] reported that selective intrahepatic depletion of CD4+ T cells robustly induced tumor development in a methionine-choline-deficient liver specific MYC transgenic mouse showing that CD4+ T cells mediate tumor regression[69]. Moreover, stimulation of hepatocellular lymphotoxin-β receptor (LTBR) and NF-kB signaling led to HCC onset in a MCD high fat diet mouse model whereas that same dietary pattern induced activation of natural killer T (NKT) cells and intrahepatic CD8+ T cells, which in turn facilitated NASH to HCC transition [70]. Additionally, liver damage and subsequent inflammatory response leads to activation of Kupffer cells (KCs), which are the resident macrophages of the liver and their involvement into NAFLD progression is well established in both animal models and human hepatic dysregulation[71]. Yet, these cells are also implicated in the hepatocarcinogenesis since they express the pro-inflammatory myeloid cell surface receptor TREM1 which facilitates HCC development in a diethyl nitrosamine-induced HCC mouse model[72]. KCs also express Toll-like receptor 4 and binding of Lipopolysaccharides drives the activation of the above-mentioned tumorigenic pathways of NF-kB, JNK and MAPK[73]. Additionally, upon acute liver cells injury, the signaling pathway Hedgehog was triggered and reinforced the recruitment of hepatic progenitor cells at the sites of injury in order to replace the damaged hepatocytes[74]. Dysregulated signaling of this pathway leads to insufficient cell repair within the hepatic parenchyma and results in malignancy and HCC progression[75].

RISK FACTORS

Although cirrhosis constitutes the major risk factor for the development of HCC in various liver diseases, including NAFLD, HCC can also occur in non-cirrhotic NAFLD individuals[76]. Demographic, behavioral, or genetic factors contribute along with cirrhosis or even more in absence of cirrhosis to HCC. Older age, male sex and Hispanic ethnicity are strongly associated with higher risk of HCC development[5]. In a cohort study of 296707 NAFLD patients, age over 65 years comprised an

Table 2 Characteristics of non-alcoholic fatty liver disease-associated hepatocellular carcinoma based on studies including cohorts of non-alcoholic fatty liver disease-associated hepatocellular carcinoma patients

Ref.	Year/Country	Total NAFLD-HCC patients	Prevalence of NAFLD-HCC without cirrhosis, *n* (%)	Fibrosis stage of non-cirrhotic NAFLD-HCC patients, *n* (%)	Tumor characteristics in cirrhotic *vs* non-cirrhotic NAFLD-HCC patients (differentiation)
Piscaglia *et al* [24]	2015/Italy	145 patients	67 (46)	F0 = 3 (18.75); F1-F2 = 2 (12.5); F3 = 11 (68.75) (Undefined fibrosis stage in 51 patients)	NSD in tumor size
Leung *et al*[46]	2015/Australia	54 patients	8 (15)	F0 = 2 (33.3); F1-F2 = 4 (66.7) (Undefined fibrosis stage in 2 patients)	↑ tumor diameter in non-cirrhotic (4.7 cm) *vs* cirrhotic (3.2 cm) (P = 0.041). NSD in median number of tumors in non-cirrhotic (2) *vs* cirrhotic (1). NSD in HCC differentiation
Kodama *et al* [47]	2019/Japan	104 patients	58 (55.8)	F0 = 6 (5.8); F1 = 11 (10.6); F2 = 18 (17.3); F3 = 23 (22.1)	NSD in HCC differentiation
Mohamad *et al*[48]	2015/United States of America	83 patients	36 (43.4)	F0 = 18 (55.9); F1 = 6 (17.6); F2 = 3 (8.8); F3 = 6 (17.6)	↑ incidence of single nodules in non-cirrhotic (80.6%) *vs* cirrhotic (52.2%) (P < 0.05). ↑ proportion of large nodule size (> 5 cm) in non-cirrhotic (77.8%) *vs* cirrhotic (10.6%) (P < 0.05). NSD in HCC differentiation
Tobari *et al* [49]	2020/Japan	119 patients	48 (40.3)	F0-F1 = 12 (32.4); F2 = 17 (46); F3 = 8 (21.6) (Undefined fibrosis stage in 11 patients)	↑ tumor size in non-cirrhotic (46 mm) *vs* cirrhotic (28 mm) (P < 0.01). NSD in HCC differentiation. NSD in median number of tumors
Yasui *et al*[25]	2011/Japan	87 patients	43 (49.4)	F1 = 10 (23.2); F2 = 15 (34.9); F3 = 18 (41.9)	NA
Thompson[50]	2018/United States	48 patients	26 (54)	F0 = 10 (38.5); F1 = 8 (30.8); F2 = 5 (19.2); F3 = 1 (3.8)	↓ tumor size in non-cirrhotic (3.3 cm) *vs* cirrhotic (5.7 cm) (P < 0.01). NSD in HCC differentiation
Cotrim *et al* [51]	2016/Brazil	110 patients	20 (48.5)	F0 = 2 (12.5); F1-3 = 14 (87.5)	NA
Iannaccone *et al*[52]	2007/France	22 patients	16 (72.3)	F0 = 7 (31.8); F1-3 = 9 (40.9)	NA

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NSD: No significant difference; NA: Not applicable.

independent risk factor for HCC occurrence[77]. In the same study, the incidence of HCC was higher in males compared to females (0.22 *vs* 0.04 *per* 1000 PY respectively), in Latino *vs* White and African-American patients (0.29 *vs* 0.21 and 0.12 *per* 1000 PY, respectively) and in cirrhotic compared to non-cirrhotic patients (10.2 *vs* 0.02 *per* 1000PY, respectively)[77].

Furthermore, distinctive MetS-related features, namely obesity and T2DM have been identified as risk factors for HCC[78]. In a meta-analysis, overweight and obese patients had 17% and 89% increased relative risk for HCC respectively compared to normal-weight individuals[79]. In another study, obesity was recognized as an independent predictor for HCC development only in patients with cryptogenic [odds ratio (OR): 11.1; 95% confidence interval (CI): 1.5-87.4] and ALD-related cirrhosis (OR: 3.2; 95%CI: 1.5-6.6)[80]. Concerning the burden of T2DM, in a retrospective study including 6508 NAFLD patients, T2DM comprised an independent risk factor for the

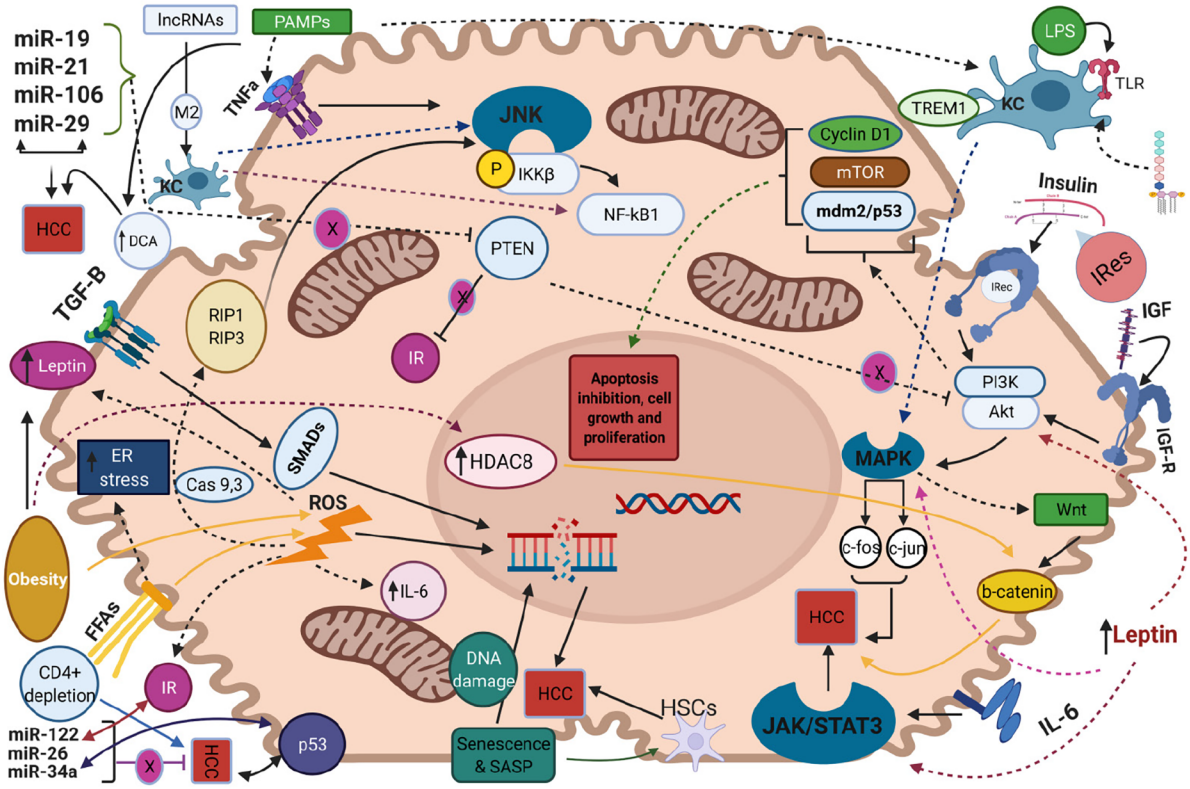


Figure 1 The molecular pathways that regulate non-alcoholic fatty liver disease-related hepatocellular carcinoma along with their interactions are represented. Activation of downstream signaling pathways is indicated by full or dot lines whereas inhibition of them is indicated by blunted lines with a circular-shaped "X". Bidirectional arrows highlight the interplay between distinct molecular pathways. Molecules acting as mediators of signaling paths are indicated over a full, dot or blunted line in a box along with their names. MicroRNAs that promote hepatocellular carcinoma development are collectively represented in the upper left of the figure while tumor suppressor microRNAs are represented in the lower left of the schema. Cas: Caspase; DCA: Deoxycholic acid; ER: Endoplasmic reticulum; FFAs: Free fatty acids; HCC: Hepatocellular carcinoma; HDAC8: Histone deacetylase 8; HSCs: Hepatic stellate cells; IGF: Insulin growth factor; IL-6: Interleukin-6; IRec: Insulin receptor; IRes: Insulin resistance; JNK: Jun-(N)-terminal kinase; KC: Kuppfer cell; lncRNAs: Long non-coding RNAs; LPS: Lipopolysaccharides; MAPK: Mitogen-activated protein kinase; PAMPs: Pathogen-associated molecular patterns; SASP: Senescence associated secretory phenotype; STAT3: Signal transducer and activator of transcription 3; TGF-B: Transforming growth factor-B; TLR: Toll-like receptor.

HCC (Hazard ratio: 3.21; 95%CI: 1.09-9.50)[81], while in a Mayo clinic study with 354 NASH-cirrhotic patients, T2DM along with older age and decreased serum albumin levels were identified as independent risk factors for HCC[82]. Consistently, in a case-control study of 185 HCC cases and 404 controls, T2DM (OR: 4.33, 95%CI: 1.89–9.86) and obesity (OR: 1.97, 95%CI: 1.03–3.79) were associated with increased HCC risk, while the combination of obesity and T2DM further exacerbated the hazard of HCC (OR: 4.75, 95%CI: 1.75-12.89)[83]. However, it should be noted that evaluating the exact independent pathogenetic burden of T2DM or obesity in the development of HCC can be really challenging, due to the strong association of these two entities[84].

Moreover, lifestyle modifiable factors, such as smoking and alcohol consumption seem to be implicated in NAFLD-related HCC[84]. In a meta-analysis of 81 studies, the risk for HCC development was higher for both current and former smokers[85], but no specific data were given regarding the relationship between smoking and the incidence of HCC in NAFLD patients[84]. Alcohol consumption is independently related to elevated risk for HCC in NAFLD patients[86], despite that some studies imply that the higher HCC risk is limited only on heavy alcohol use (*e.g.* > 50 g/d[87, 88]), during which NAFLD is excluded by definition. Intriguingly, Sookoian *et al*[89] in their meta-analysis, showed that moderate alcohol consumption (< 30 g/d) is associated with decreased incidence of NAFLD occurrence[89], whereas a prospective study demonstrated a potential synergism between obesity and alcohol intake in increasing the risk of HCC[90]. However, the evidence from those studies could be hindered by potential biases, such as the observational study design which does not permit to ascertain causality, the implication of obesity as potential confounder and the overall insufficient alcohol intake assessment. Overall, the impact of moderate alcohol consumption in NAFLD-related HCC is still difficult to be clarified.

Noteworthy, genetic predisposition further aggravates the risk for NAFLD-related HCC. The possession of Patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409C>G and Membrane Bound O-Acyltransferase Domain Containing 7rs641738 polymorphism were independently correlated with increased risk of HCC [91], with the latter being a burdened factor particularly in non-cirrhotic NAFLD patients[92]. On the contrary, a loss of function of variant rs72613567 in 17-beta-hydroxysteroid dehydrogenase 13 has been recently identified to protect against HCC development[93].

PROGNOSIS OF NAFLD-RELATED HCC

The prognostic outcomes of NAFLD-HCC patients as compared to their non-NAFLD-HCC counterparts have been evaluated by population-based and cohort studies with controversial findings (Table 3)[15,24,30,34,40,42,94-103].

Based on Surveillance, Epidemiology and End Results Medicare database, Younossi *et al*[30] performed a large retrospective cohort study including 4979 HCC patients and showed that the one-year mortality risk was significantly higher in NAFLD-HCC compared to HCV/HBV-HCC patients[30]. On average, the former had almost 5 mo shorter survival time compared to the latter[30]. Moreover, in the multivariable model adjusted for clinical and tumour-related parameters, NAFLD was an independent factor associated with increased one-year mortality risk[30]. More recently, Golabi *et al* [94] based on the afore-mentioned database along with outpatients files, showed that NAFLD-HCC patients displayed markedly higher risk of 2-year mortality in comparison with patients with HCV-HCC[94]. Consistent to the abovementioned study, the magnitude of this association remained significant in the multivariable analysis[94]. The afore-mentioned findings were mainly attributed to the more advanced tumour stage at the time of diagnosis due to less intense surveillance of NAFLD patients[94]. In addition, due to the presence of increased visceral obesity, the ultrasonography, which is the current HCC screening tool, may fail to distinguish small tumours in NAFLD patients[94]. Furthermore, the increased prevalence of cardio-metabolic comorbidities along with higher age at the time of HCC diagnosis of NAFLD population may also contribute to the lower possibility of receiving LT, compared to HCC patients with viral hepatitis, and to their decreased survival time [94]. In a prospective study in Italy, among 756 HCC patients the crude 1-year and 3-year overall survival (OS) was remarkably lower in the NAFLD-HCC patients compared to HCV-HCC cohort[24]. In order to account for the potential biases of less intense surveillance and later detection of the NAFLD-related malignancy, they adjusted the whole HCC cohort of patients for the lead time who were under surveillance[24]. Intriguingly, both the mean 1-year and 3-year survival time of NAFLD-HCC patients remained significantly lower than the corresponding of HCV-HCC patients[24]. Along this line, more recently, Hester *et al*[96] in their multivariable analysis demonstrated that the NASH-HCC was associated with worse OS compared only to the ALD-HCC[96]. Additionally, since the magnitude of NAFLD in cryptogenic cirrhosis is major, it is of interest that Giannini *et al*[97] using data form the ITALICA database, showed that patients with cryptogenic cirrhosis-related HCC displayed significantly shorter survival time compared to HCV-HCC patients during a median follow-up of 21 mo[97].

Of importance, we should note that a selection bias within clinical studies could be implicated concerning the worse prognostic outcomes of NAFLD-HCC patients in comparison to other-aetiologies-HCC. Patients who were eligible for radical surgical treatments or LT, were subsequently enrolled in the cohort studies whereas patients with major comorbidities, cardiovascular diseases or advanced age, aspects more common among NAFLD patients, were excluded.

On the contrary, numerous studies have emphasized the better prognosis of NAFLD-HCC patients, compared to other aetiologies of HCC. In a prospective study from Singapore, 844 non-NAFLD-HCC and 152 NAFLD-HCC patients who underwent total liver resection were enrolled and the latter displayed significantly increased 5-year OS as compared to the former, whereas NAFLD was independently associated with lower hazard for mortality in a multivariable model adjusted for clinical and epidemiological parameters[34]. Consistently, after a median follow-up of 50 mo, Reddy *et al*[42] evaluated HCC patients suffered from NASH compared to those from ALD and/or HCV who received curative treatment[42]. Although the postoperative mortality and the recurrence free survival (RFS) did not significantly differ between the two groups, NASH patients had longer OS, compared to ALD and

Table 3 Treatment outcomes and prognosis in patients with non-alcoholic fatty liver disease-related hepatocellular carcinoma *vs* other etiologies-related hepatocellular carcinoma

Ref.	Year/country/type of study	Total HCC patients (underlying disease)	Features of hepatocellular carcinoma (NAFLD *vs* other etiologies)	Treatment	Overall survival (NAFLD *vs* other etiologies)	Recurrence-free survival (NAFLD *vs* other etiologies)
Younossi *et al*[30]	2015/United States/Retrospective	4979 with HCC; 701 NAFLD, 254 AH/BC, 817 ALD, 471 HBV, 2736 HCV	NAFLD: ↑ possibility of unstaged HCC *vs* HCV/HBV	LT	NAFLD: ↓ OS *vs* HCV/HBV (NAFLD: 1-yr mortality risk is 61% *vs* 50% for the HCV/HBV group)	NA
Golabi *et al*[94]	2017/United States/Retrospective	11187 total HCC patients; 1277 NAFLD, 1421 ALD, 586 HBV, 3591 HCV	Among HCC patients treated with SR: 57% had HCV *vs* 17% had NAFLD	LT, SR, TACE	NAFLD: ↓ OS *vs* HCV and/or HBV (HR: 0.82) but ↑ OS *vs* ALD (HR: 1.59)	NA
Piscaglia *et al*[24]	2016/Italy/Prospective	756 total HCC patients; 145 NALFD, 611 HCV	NAFLD: More advanced BCLC HCC stage and more commonly outside the Milan criteria *vs* HCV	LT, SR, PEI, Thermal ablation, TACE, BSC or trials	NAFLD: ↓ 1-yr and 3-yr OS *vs* HCV (1-yr and 3-yr survival; 76.4% and 48.7% in the NAFLD-HCC group and 84.2% and 61.1% in the HCV-HCC respectively) NSD among treatment choices	NA
Hester *et al*[96]	2019/United States/Retrospective	1051 total HCC patients; 92 NASH, 153 ALD, 87 HBV, 719 HCV	NASH and HBV HCC patients: Larger median tumor size *vs* HCV and ALD. NSD in BCLC staging among the groups	LT, SR or ablative techniques, TACE, yttrium 90, or TARE or radiation therapy, systemic therapy	NAFLD: ↓ OS *vs* ALD (HR: 1.92) NSD between NAFLD-HCC and viral-related HCC	NA
Giannini *et al*[97]	2009/Italy/Prospective	471 total HCC patients; 45 CC, 426 HCV	CC: ↑ prevalence of multinodular and diffuse lesions, ↑ size of the largest lesion and advanced classification according to Milano criteria (69% *vs* 41%) *vs* HCV	LT, SR, PEI, RFA, TACE	CC: ↓ OS *vs* HCV	NA
Koh *et al*[34]	2019/Singapore/Prospective	996 total HCC patients; 152 with NAFLD, 844 non-NAFLD	NAFLD: Smaller median tumor size	Total liver resection	NAFLD: ↑ 5-yr and 10-yr OS *vs* non-NAFLD groups (5-yr and 10-yr OS; 70.1% and 49.6% in the NAFLD-HCC group *vs* 60.9% and 41.0% in the non-NAFLD-HCC respectively)	NSD in RFS (P = 0.0931)
Reddy *et al*[42]	2012/United States/Retrospective	303 HCC patients; 52 with NAFLD *vs* 162 HCV/ALD	NASH: NSD in largest tumor size, tumor differentiation and presence of satellite lesions *vs* HCV/ALD	Resection, ablation, and LT	NASH: ↑ 3-yr OS *vs* HCV/ALD (60.9% *vs* 36.2%)	NSD
Benhammou *et al*[98]	2020/United States/Retrospective	454 total HCC patients; 125 NAFLD, 170 HBV, 159 HCV	NAFLD and HCV more likely to be within Milan and UCSF criteria for LT *vs* HBV	LT, SR, RFA, PEI, TACE/Y-90, chemotherapy, BSC	NAFLD: ↑ OS *vs* HBV (HR: 0.35) and HCV (HR: 0.37)	NAFLD: ↑ RFS *vs* HCV (HR: 0.64) and HBV (HR: 0.69)
Viganò *et al*[99]	2015/United States/Retrospective	1563 total HCC patients; 96 HCV, 96 MetS matched	MetS: NSD in satellite nodules and microvascular invasion *vs* HCV	SR, preoperative PVE, TACE	MetS: ↑ OS *vs* HCV (65.6% *vs* 61.4%)	MetS: Trend for ↑ RFS *vs* HCV (37.0% *vs* 27.5%, P = 0.077)
Bengtsson *et al*[40]	2019/Sweden/Retrospective	1562 total HCC patients; 225 NAFLD, 1337 non-NAFLD	NAFLD: NSD in BCLC staging, number of tumors and largest tumor size *vs* non-NAFLD	LT ± RFA or TACE, SR, RFA, TACE, systemic therapy or BSC	NAFLD: NSD in OS *vs* non-NAFLD (HR: 1.04)	NA
Than *et al*[100]	2017/United States/Retrospective	487 total HCC patients; 212 NAFLD, 275 HCV	NAFLD: ↑ tumor size *vs* HCV	TACE, RFA, SR, PEI, sorafenib, LT	NAFLD: NSD *vs* HCV (44% *vs* 56% respectively)	NA

Wakai *et al*[101]	2011/Japan/Retrospective	225 total HCC patients; 17 NAFLD, 61 HBV, 147 HCV	NAFLD: ↑ tumor size *vs* HCV & HBV	SR	NAFLD: ↑ postoperative morbidity and 30-d mortality rates (59% and 12% in NAFLD *vs* 31% and 0.7% in HCV respectively & 28% and 3.3% in HBV respectively)	NAFLD: ↑ RFS *vs* HBV & HCV
Jung *et al*[95]	2021/South Korea/Retrospective	426 total HCC patients; 32 NAFLD, 200 HBV, 194 HBV/NAFLD	NAFLD: ↑ average tumor size *vs* HBV group (4.4 cm *vs* 3.4 cm)	Hepatectomy	Before PSM: NAFLD: ↓ 5-yr OS *vs* HBV (63% *vs* 80%). After PSM, NSD in 5-yr OS rates	NSD in RFS or disease-specific survival before and after PSM
Tokushige *et al* [102]	2010/Japan/Prospective	90 total HCC patients; 34 NASH, 56 HCV	NASH: NSD in tumor size *vs* HCV	SR, RFA, TACE	NASH: NSD in 5-yr survival rate (55.2% in NASH *vs* 50.6% in all HCV)	NSD in 5-yr recurrence rate
Pais *et al*[15]	2017/France/Retrospective	323 total HCC patients; 39 NAFLD, 284 non-NAFLD	NAFLD: ↑ larger tumor size *vs* non-NAFLDNSD in other tumor characteristics	SR, TACE, PVE, PEI, LT	NSD in 2.5 post-LT OS (Mortality: 36% in NAFLD, 48% in ALD, 45% in HCV and 36% in CHB)	NSD
Hernandez-Alejandro *et al* [103]	2012/Canada/Retrospective	81 total HCC patients; 17 NASH, 64 HCV	NASH: ↓ proportion had poorly differentiated HCC *vs* HCV	LT	NA	NASH: trend of ↑ 5-yr RFS (P = 0.11)

HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; AH/BC: Autoimmune hepatitis/Biliary cirrhosis; ALD: Alcohol-related liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; LT: Liver transplantation; OS: Overall survival; NA: Not applicable; SR: Surgical resection; TACE: Transarterial chemoembolization; BCLC: Barcelona-Clinic Liver Cancer; PEI: Percutaneous ethanol injection; BSC: Best supportive care; NSD: No significant difference; RFS: Recurrence-free survival; NASH: Non-alcoholic steatohepatitis; TARE: Transarterial radioembolization; HR: Hazard ratio; CC: Cryptogenic cirrhosis; RFA: Radiofrequency ablation; MetS: Metabolic syndrome; PVE: Portal vein embolization; OR: Odds ratio; UCSF: University of California at San Francisco.

HCV patients, independently of clinical factors and type of the curative treatment they received[42]. In another study, during a median follow-up of 17 mo, NAFLD-HCC patients displayed significantly improved OS and a trend towards increased RFS, compared to both HCV and HBV patients, in a model adjusted for demographic factors, Child-Pugh score and most definite treatment[98]. Notably, in order to assess the afore-mentioned long-term outcomes independently of the LT, authors omitted the LT recipients from all groups[98]. To this end, they showed that the NAFLD-HCC patients still had significantly improved OS rates compared to their HCV counterparts and a trend towards increased survival compared to HBV patients[98]. In 2015 Viganò *et al*[99], matched 96 HCC patients with MetS with 96 HCV-HCC patients who received liver resection during a 12-year study period[99]. Matching was based on age, prevalence of cirrhosis, Child-Pugh class, portal hypertension and HCC characteristics [99]. MetS-HCC patients had significantly better OS and lower recurrence rate compared to HCV-HCC cases whereas in the multivariate analysis MetS-HCC was an independent protective factor for both OS and early recurrence[99].

Finally, several studies have highlighted the similar long-term outcomes regarding NAFLD and non-NAFLD-HCC patients. A Swedish retrospective study revealed that although NAFLD-HCC patients had higher age, higher prevalence of comorbidities and less HCC surveillance, they had similar survival to non-NAFLD-HCC patients, mainly attributed to the poor prognosis of HCC in general[40]. Consistently, Than *et al* [100] compared the outcomes of 212 NAFLD-HCC and 275 HCV-HCC patients who

were referred for LT and showed that the 3-year post-diagnosis OS was similar in the two groups[100] and this finding was confirmed in the subgroup-analysis of patients who eventually received LT[100]. Along this line, Wakai *et al*[101] upon evaluating 317 HCC patients who received hepatic resection, showed that the 5-year post-resection cumulative survival rate did not differ significantly between NAFLD and non-NAFLD groups. Yet, RFS following liver resection was markedly better in the NAFLD group [101]. Additionally, Jung *et al*[95] reviewed the outcomes of NAFLD-HCC and HBV-HCC patients who underwent hepatectomy over a 10-year period. After a median follow up of 74 mo, the latter had superior 5-year OS rates, compared to the former. However, when the authors performed a propensity score matching to minimize the bias of lead time, 5-year OS was similar between the two groups[95]. In a Japanese prospective study, the OS and the RFS after curative treatment of NAFLD-HCC and HCV-HCC patients were evaluated, and both were comparable between the two cohorts[102]. Similarly, Pais *et al*[15] in their retrospective study evaluating a 20-year period confirmed those results[15], while a Canadian study revealed only a trend for better 5-year RFS of NASH-HCC, compared to HCV-HCC patients who underwent LT [103].

However, we should emphasize that due to less intense surveillance of HCC in general, the disease is likely to be diagnosed at advanced stages and therefore even fewer patients are eligible for radical therapy or LT. Thus, the prognostic outcomes of NAFLD-HCC patients compared to non-NAFLD-HCC ones, should be interpreted with cautiousness and accordingly to each clinical study design and type of HCC-treatment that patients received.

Moreover, another factor raising concern for defining the long-term survival outcomes of NAFLD-HCC patients is that those patients seem to have a lower MELD score waiting at LT list compared to their non-NAFLD counterparts, as they tend to have a more preserved liver function. Therefore, they are less likely to receive LT in short-term. This in turn results in longer duration in the waiting list increasing the risk for severe health-related complications and morbidity negatively affecting their survival. Indeed, when Wong *et al*[104] retrospectively analysing UNOS registry data concerning LT waiting list registrations in the United States, demonstrated that NAFLD patients as compared to their HCV or ALD counterparts, were markedly less likely to receive a liver transplant within 90 d and one year after their registration, ending in higher mortality while on the waiting list[104].

SURVEILLANCE

Since NAFLD patients seem to be frequently diagnosed in advanced tumor stages, their surveillance for HCC development represents quite the most challenging issue among professional societies worldwide. As for the cirrhotic-NAFLD patients, since they appear to have an expected HCC incidence of approximately 1.5% *per* year, they should follow the screening guidelines for cirrhotic patients of any cause, consisted of abdominal examination with liver ultrasonography with or without alpha-fetoprotein (AFP) every 6 mo[105] (Figure 2).

Regarding non-cirrhotic NAFLD patients, there is a lack of consensus whether NAFLD patients with F3 fibrosis should undergo screening. The updated recommendation of EASL guidelines suggest that patients with F3-fibrosis might be eligible for HCC surveillance based on an individual risk stratification[106], while the clinical update of the American Gastroenterology Association also recommend the screening for patients with findings indicative of advanced fibrosis (F3), as evaluated by two or more concordant non-invasive fibrosis tests of separate categories[107]. AASLD guidelines recommend HCC surveillance only in cirrhotic (not advanced fibrosis-F3) patients[108]. Finally, the Asian guidelines do not provide specific recommendation for non-cirrhotic NAFLD patients[109] (Figure 2). However, some concerns are raised. Even if a consensus for screening of F3 patients was reached, a large proportion of HCC cases that occur in F0-F2 NAFLD patients would still be missed. Moreover, the diagnosis of F3 fibrosis based on a broad spectrum of non-invasive tests mitigates the utility of screening since HCC risk would not still be the same for all F3-patients, while screening all patients with F3 fibrosis would drastically increase the cost of the surveillance strategy. Moreover, it should be mentioned that even among cirrhotic patients, there are differences in risk for HCC and therefore they should not be aggregated into a single category. Ioannou *et al*[110] developed a predictive model that estimates HCC risk in cirrhotic-NAFLD patients by implicating demographic, clinical and laboratory parameters[110]. Based on this model, patients

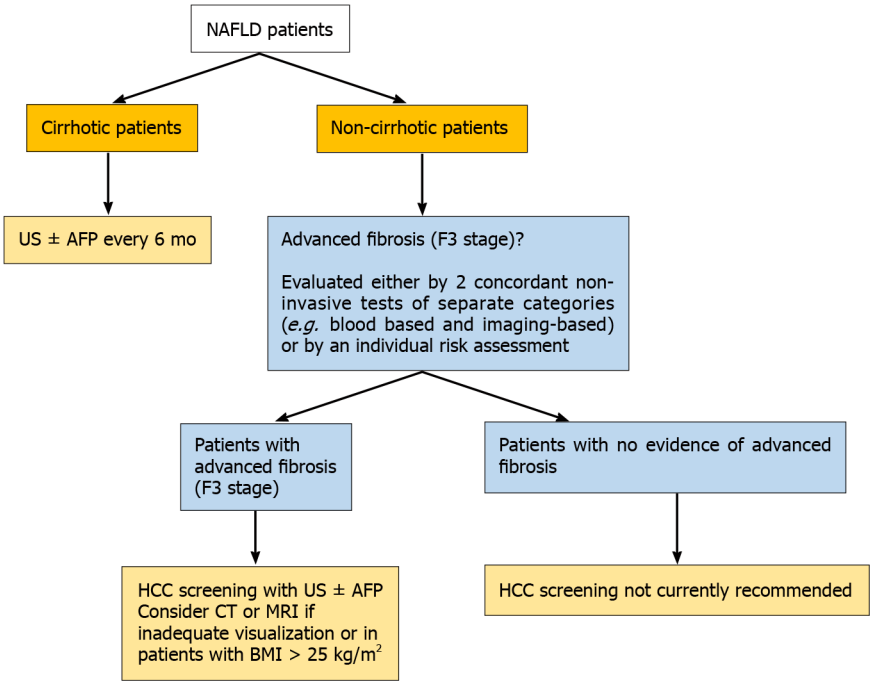


Figure 2 Proposed algorithm for hepatocellular carcinoma surveillance in non-alcoholic fatty liver disease patients based on the latest guidelines. HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; US: Ultrasonography; AFP: Alpha-fetoprotein; CT: Computer tomography; MRI: Magnetic resonance imaging; BMI: Body mass index.

were categorized into low-risk (annual risk: < 1%), medium-risk (annual risk: 1%–3%) and high-risk (annual risk: > 3%), suggesting that individualized screening for HCC is associated with standardized benefit compared with screening of all cirrhotic-NAFLD patients[110,111]. Moreover, the identification of PNPLA3rs738409C>G or other polymorphisms might be of value for screening since it would improve prediction accuracy, but its evaluation in multi-factorial risk models would decrease the cost-effectiveness of patients' surveillance[112]. The future surveillance policies should focus on identification of prognostic factors, consisting of imaging modalities, serum biomarkers and genetic variants, testing for which would need to become cheaper, that will stratify the risk of HCC in both cirrhotic and non-cirrhotic NAFLD patients promoting the cost-effectiveness of the programmes. The afore-mentioned parameters along with well-established traditional risk factors of HCC may be incorporated in future risk-assessment models and could result in more accurate prediction of NAFLD-related HCC and optimized surveillance strategies. A proposed algorithm of NAFLD-related HCC surveillance based on future perspectives is illustrated in Figure 3.

As yet, circulating micro-RNAs (miRNAs) and long non-coding-RNAs have been shown promising results since they were associated with HCC progression in NAFLD patients and may constitute potential non-invasive tools for NAFLD-related HCC screening[113,114]. Several micro-RNAs, such as miR-29 and miR-199, mainly expressed in NASH, are associated with fibrosis progression and HCC development [113]. Furthermore, hydroxy-methylated genes are strongly related to the involvement of chromatin in the progression of HCC and form promising genetic factors for the risk classification of AFP-negative HCC patients[115]. Results derived from animal models examining the progression of HCC in NASH mice, suggested that serum osteopontin and dikkopf-1 could be possible novel biomarkers for the early detection of HCC[116]. Finally, the identification and amplification of circulating tumor-DNA can reveal critical HCC-related genetic mutations and therefore could be used for the screening of HCC patients[117]. However, the incorporation of those prognostic biomarkers in screening programmes would significantly increase the cost with ambiguous results. As until now, they comprise mostly future perspectives rather than clinical point-of-care practice.

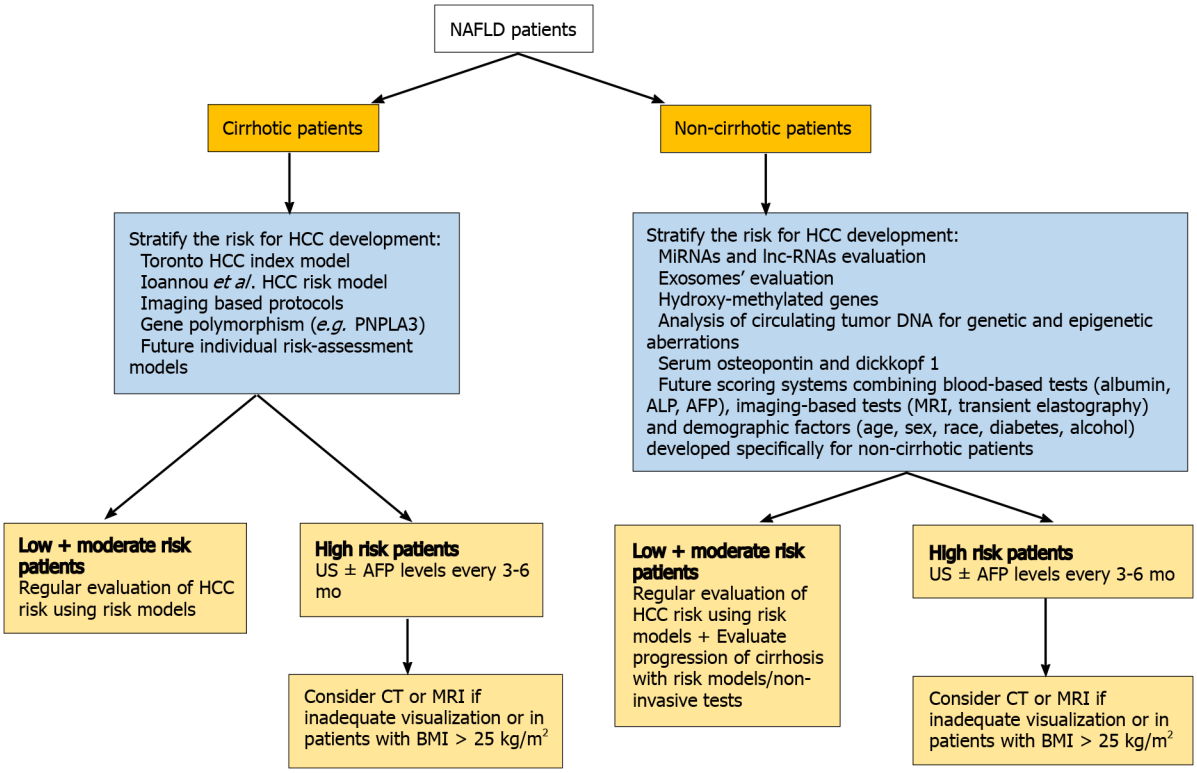


Figure 3 Proposed algorithm for hepatocellular carcinoma surveillance in non-alcoholic fatty liver disease patients based on future perspectives. HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; PNPLA3: Patatin-like phospholipase domain-containing protein 3; MiRNAs: Micro-RNAs; lnc-RNAs: Long non-coding-RNAs; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; US: Ultrasonography; CT: Computer tomography; MRI: Magnetic resonance imaging; BMI: Body mass index.

PREVENTION

Although weight loss is considered fundamental for the management of NAFLD, there is no current evidence to directly indicate that weight loss leads to reduction of NAFLD-related HCC. Importantly, a recent large multi-national study with 467336 individuals, demonstrated that physical exercise, defined as performing at least 2 hours of vigorous activity *per* week, can reduce the risk of developing HCC independently of other risk factors of HCC[118]. Moreover, the prevention of obesity and T2DM is considered fundamental for the management of NAFLD patients, since they constitute independent risk factors for HCC development and progression[80,82,111,119]. Noteworthy, a meta-analysis of 19 studies showed that diet rich in vegetables may reduce HCC incidence, while George *et al*[120] suggested that stricter adherence to Mediterranean diet was protective against HCC development[120]. Increased coffee consumption is also associated with decreased risk of NAFLD development and severity progression[121], with two additional coffee cups *per* day to be associated with 35% lower incidence of HCC[122]. However, the exact impact of coffee consumption as a preventive measure against NAFLD and its progression to HCC needs further investigation. Although literature data are controversial regarding the role of light and moderate alcohol use in NAFLD *per se*[89,90], in a study of 195 cirrhotic-NASH patients, Ascha *et al*[86] demonstrated that patients with any alcohol consumption had higher risk of HCC incidence compared to non-drinkers[86]. Consistently, HCC occurrence was more frequent in NAFLD patients with mild alcohol intake (< 20 g/d), especially in those with advanced fibrosis, as compared to abstainers patients[123]. Concerning medication, in a prospective study of 361 NAFLD patients, daily use of aspirin was associated with significant lower odds for NASH and advanced fibrosis, while no relationship between use of non-aspirin nonsteroidal anti-inflammatory drugs and risk for advanced fibrosis was outlined[124]. Furthermore, the administration of statins in cirrhotic patients provides chemo-preventive effects and is associated with the reduction of HCC occurrence, in a dose-dependent manner [125-127]. Interestingly, fluvastatin, compared with other statin interventions, exhibited the most significant effect in the reduction of HCC incidence in cirrhotic patients, while the utility of rosuvastatin against the development of NAFLD-related

HCC was shown in a murine model[127,128]. As far for T2DM pharmacotherapy, dose-response anti-tumorigenic effects of metformin were observed among T2DM patients[129], while in contrast, the administration of sulfonylureas and insulin have been associated with increased risk of HCC[129].

CONCLUSION

In the 21st century, we are in the midst of an epidemic of obesity, T2DM and NAFLD. Consequently, the burden of NAFLD in HCC development is rapidly rising partially explaining the elevated incidence of HCC in both men and women globally. Although the exact pathogenetic mechanisms involved in NAFLD-related HCC onset are still not well-established especially regarding the non-cirrhotic hepatic parenchyma, specific risk factors for HCC concerning demographic, genetic and behavioral parameters have been already identified. Noteworthy, the surveillance of NAFLD-HCC patients is not standard in medical practice and therefore many patients do not undergo screening and that leads to diagnosis of HCC at advanced stages negatively affecting their survival and diminishing the therapeutic options. Concerning systemic treatment for HCC, the latest data[130,131] do not support the hypothesis that the therapeutic decisions should be based on the underlying HCC etiology and therefore, HCC systemic therapy was not in the field of our review. However, noteworthy, in a recent meta-analysis of 3 trials[132-134], authors suggested that immunotherapy might be less efficacious in NASH-HCC patients as compared to their viral-HCC counterparts, presumably owing to the NASH-provoked aberrant T-cells activation and subsequently flawed immune surveillance[135]. Yet, more robust evidence are needed for therapeutic decision making. Although, lifestyle modifications, such as stricter adherence to Mediterranean diet and medication namely metformin are thought to contribute to the primary prevention of NAFLD-related HCC, the appropriate strategy would be the identification of at-risk patients *via* a relatively simple score including demographic and laboratory/imaging parameters. Implementation of risk stratification programmes and high awareness of the burden of NAFLD should be the primary goals for medical clinical specialties and health authorities worldwide.

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MINIREVIEWS

Endoscopic ultrasound role in pancreatic adenocarcinoma treatment: A review focusing on technical success, safety and efficacy

Wisam Sbeit, Bertrand Napoléon, Tawfik Khoury

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

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Wisam Sbeit, Tawfik Khoury, Department of Gastroenterology, Galilee Medical Center, Faculty of Medicine in the Galilee, Bar-Ilan University, Nahariya 2221006, Israel

Bertrand Napoléon, Department of Endoscopy Unit, Private Hospital Jean Mermoz, Ramsay Generale de Sante, Lyon 69008, France

Corresponding author: Tawfik Khoury, MD, Doctor, Lecturer, Senior Researcher, Department of Gastroenterology, Galilee Medical Center, Faculty of Medicine in the Galilee, Bar-Ilan University, Meona Street, Nahariya 2221006, Israel. tawfik.khoury.83@gmail.com

Abstract

The impressive technological advances in recent years have rapidly translated into the shift of endoscopic ultrasound (EUS) from diagnostic modality into an interventional and therapeutic tool. Despite the great advance in its diagnosis, the majority of pancreatic adenocarcinoma cases are inoperable when diagnosed, thus demanding alternative optional therapies. EUS has emerged as an easy, minimally invasive modality targeting this carcinoma with different interventions that have been reported recently. In this review we summarize the evolving role of interventional therapeutic EUS in pancreatic adenocarcinoma management.

Key Words: Endoscopic ultrasound; Pancreas; Cancer; Management; Palliative

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Core Tip: The prognosis of pancreatic adenocarcinoma is poor in advanced stages. Several studies were conducted recently to assess the effect of different treatment options provided through endoscopic ultrasound (EUS). We present a comprehensive review on the role of EUS in unresectable pancreatic adenocarcinoma treatment while exploring its effect on survival and palliation. We found that EUS-guided intervention is feasible with excellent technical success, limited adverse events, a beneficial effect on cancer-associated pain and an as-yet unknown effect on survival. For EUS-assisted therapies there are still many unknowns and unanswered questions, prompting the need for additional prospective randomized controlled studies comparing the different

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INTRODUCTION

Pancreatic adenocarcinoma is the seventh leading cause of cancer death worldwide with poor prognosis according to the 2020 GLOBOCAN cancer estimates[1]. About half of patients are diagnosed with metastatic disease and 30% with locally advanced disease and are deprived from the only potential cure of surgical intervention[2]. The median overall survival for stages IV and III is of 2-3 and 7-11 mo, respectively[3]. As a result, those patients are usually offered supportive care, palliative chemotherapy and radiology, and palliative surgical interventions. Endoscopic ultrasound (EUS), first introduced about 40 years ago as a diagnostic tool, has quickly gained popularity as an interventional therapeutic tool in a broad range of gastrointestinal, pancreato-biliary and liver diseases due to its high spatial resolution. There are several characteristics of EUS that improve its utility as an interventional therapeutic instrument. The first and most crucial property is its high spatial resolution and the proximity of its transducer to the target lesion, allowing it to access small lesions while avoiding intervening structures, blood vessels and air[4]. The second advantage lies in its minimal invasiveness and high safety profile in targeting pancreatic lesions; these have advanced this modality over interventional radiology and surgery in diverse pancreatic tumor treatment applications[5]. The third advantage is its ability to obtain contrast-enhancement images which seems to improve diagnostic performance in pancreatic masses[2]. The final benefit is the technical advancement in developing devices designed specifically to allow minimally invasive therapeutic interventions[6]. An increasing number of articles reporting these new EUS applications in pancreatic adenocarcinoma have been published, including EUS guided thermal ablation, ethanol ablation, delivery of antitumor agents, brachytherapy, fiducial marker placement (FMP), and EUS-guided celiac plexus neurolysis/block (CPN/B). In this review we summarize the literature dealing with interventional therapeutic EUS in pancreatic adenocarcinoma, aiming to present an updated comprehensive review on this topic.

LITERATURE SEARCH

A search for studies published before August 2021 was performed in the PubMed databases with the keywords EUS or endoscopic ultrasound and any of the following: Carcinoma or adenocarcinoma of pancreas, pancreatic tumor, treatment or therapeutic, intervention, ablation, injection, brachytherapy, fiducial markers and CPN. The search was restricted to articles in the English language and included prospective, retrospective, case series and randomized controlled studies. Review articles and case reports were not included. Subsequently, we generated a state-of-the-art comprehensive review by summarizing the most updated data on EUS-guided intervention published in the last several years and focusing on feasibility, technical success, safety and effect on overall survival and palliation when the data were available.

EUS-GUIDED INTRA-TUMORAL INJECTIONS

Intra-tumoral EUS fine needle injection (EUS-FNI), is a relatively new treat-to-target modality aiming to deliver and potentially achieve high intra-tumor drug concentration while minimizing systemic exposure and toxicity from those drugs[7,8]. This

method allows tumor reduction prior to surgery or serves as a palliative treatment in unresectable tumors with mass effect including obstructive symptoms[8]. EUS-FNI enables performance of several therapeutic interventions including chemotherapy, immunotherapy, gene therapy and intra-tumoral implantation. Table 1 demonstrates all studies of EUS-guided intra-tumoral injections.

CHEMOTHERAPY

A prospective study from Mayo Clinic evaluated EUS-FNI of gemcitabine in 36 patients (long-term data were available in 28 patients) with unresectable pancreatic adenocarcinoma (3 patients with stage II, 20 with stage III and 13 with stage IV). They reported no adverse events, partial response in 25% of patients, stable disease in 57% of patients and down-staging in 20% of stage III patients who underwent surgical resection, with a median of an overall survival of 10.4 mo (95% confidence interval, 2.7-68), and an overall survival of 78%, 44%, and 3% at 6 mo, 12 mo and 5 years, respectively, leading the authors to conclude that this treatment option is feasible, safe, and potentially effective[9].

IMMUNOTHERAPY

Intra-tumoral immunotherapy, including mixed lymphocyte culture and immature dendritic cells, have the ability to induce a tumor-specific immune response which can be effective, not only locally but also on metastatic lesions[7]. The first clinical trial of immunotherapy was published about 20 years ago and enrolled 8 patients with unresectable pancreatic adenocarcinoma who were treated by EUS-FNI of mixed lymphocyte culture (cytoimplant). They showed this treatment option to be feasible without procedure-related complications and with no substantial toxicity. Notably, the median overall survival was 13.2 mo, with tumor response ranging from 'minor' until 'no change'; however, there were no cases of significant or complete tumor response [10]. Later, Irisawa *et al*[11] reported their experience with seven patients suffering from stage IV gemcitabine non-responsive pancreatic adenocarcinoma who underwent EUS-FNI of immature dendritic cells with radiation therapy administered first in five patients. They showed clinical response in three of the patients with no procedure-related adverse event nor dendritic cell-related toxicity, and with an overall median survival rate of 9.9 mo[11]. Another study from Japan evaluated the feasibility, safety and histological change of preoperative EUS fine-needle injection of immature dendritic cells with OK-432 (immune-potentiating agent) in pancreatic cancer patients. In their study, nine patients were enrolled and compared to a group of 15 patients who were operated without dendritic cell injection. They reported no adverse reaction following injection in the nine patients except for one with transient fever, and no significant difference in postoperative complication incidence between both groups or in the overall median survival. Interestingly, two patients in the injection group survived for more than 5 years without disease recurrence. Analysis of resected specimens in the injection group showed that CD83 + cells significantly accumulated in the regional lymph nodes, as well as Foxp3 + cells in the regional and distant lymph nodes[12].

GENE THERAPY

Gene therapy takes advantage of the preference of oncolytic attenuated adenovirus [ONYX-015 (Onyx Pharmaceuticals, United States)] to selectively replicate in malignant cells, leading to their lysis and death[13]. The first study was performed by Hecht *et al*[14] who enrolled 21 patients with locally advanced pancreatic adenocarcinoma in a trial of intra-tumoral ONYX-015 injection *via* EUS in combination with gemcitabine. They demonstrated the feasibility, safety and tolerability of this treatment modality when EUS-FNI was performed through a trans-gastric route with prophylactic antibiotic. However, no convincing evidence of efficacy was shown as only two patients showed partial regression, two showed minor response, and six had stable disease, while 11 had progressive disease, with a median overall survival of 7.5 mo [14]. Furthermore, a subsequent study by Senzer *et al*[15] demonstrated the safety and efficacy of intra-tumoral injection of TNFerade (GenVec Inc, United States), an

Table 1 Human studies reporting endoscopic ultrasound-guided intra-tumoral injection therapies

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation	Serious adverse events, *n*
Levy *et al*[9], 2017	Prospective	II (*n* = 3); III (*n* = 20); IV (*n* = 13)	Chemotherapy	36	100	10.4	Not reported	0
Chang *et al* [10], 2000	Prospective	II (*n* = 4); III (*n* = 3); IV (*n* = 1)	Immunotherapy	8	100	13.2	Not reported	0
Irisawa *et al* [11], 2007	Prospective	IV (*n* = 7)	Immunotherapy	7	100	9.9	Not reported	0
Endo *et al*[12], 2012	Prospective	II (*n* = 1); III (*n* = 5); IV (*n* = 3)	Immunotherapy	9	100	18	Not reported	3[1]
Buscail *et al* [23], 2015	Prospective	III (*n* = 13); IV (*n* = 9)	Gene therapy	22	100	12.6	Not reported	0
Hecht *et al* [14], 2003	Prospective	III (*n* = 9); IV (*n* = 21)	Gene therapy	21	100	7.5	Not reported	4[2]
Hecht *et al* [16], 2012	Prospective	III (*n* = 27)	Gene therapy	27/50	100	9.9	Not reported	40[3]
Herman *et al* [17], 2013	Prospective	III (*n* = 95)	Gene therapy	95/187	100	11.5	Not reported	48[4]
Hanna *et al* [18], 2012	Prospective	Unresectable	Gene therapy	6	100	6	Not reported	1[5]
Hirooka *et al* [20], 2018	Prospective	III (*n* = 9)	Gene therapy	9	100	15.5	Not reported	2[6]
Nishimura *et al*[22], 2018	Prospective	III (*n* = 5); IV (*n* = 1)	Gene therapy	6	100	5.8	Not reported	0
Golan *et al* [25], 2015	Prospective	III (*n* = 15)	Intra-tumoral implantation	15	100	15.1	Not reported	4[7]

[1]Pancreatic fistula (2 patients) and superior mesenteric artery pseudoaneurysm (1 patient).
[2]Sepsis (2 patients), duodenal perforations (2 patients).
[3]The authors did not state whether these adverse events were in the endoscopic ultrasound (EUS) group or in the percutaneous group: Gastrointestinal bleeding (6 patients), deep vein thrombosis (6 patients), pulmonary embolism (2 patients), pancreatitis (2 patients), cholecystitis (1 patient), biliary obstruction (8 patients), cholangitis (6 patients), hypotension (2 patients), bradycardia (1 patient), supraventricular tachycardia (1 patient), splenic artery thrombosis (1 patient), intestinal ischemia (1 patient), staphylococcus infection (1 patient), cerebrovascular accident (1 patient), cardio-pulmonary arrest (1 patient).
[4]The authors did not state what are the serious adverse events and whether these adverse events were in the EUS group or in the percutaneous group.
[5]Hypoglycemia (1 patient).
[6]Perforation of duodenum (1 patient) and hepatic dysfunction 1 patient), but these events were considered not to be related.
[7]Colonic obstruction (1 patient), pancreatitis (1 patient), cholangitis (1 patient), renal failure (1 patient).

adenovirus vector with replication deficiency that carries the human tumor necrosis factor-alpha gene regulated by a radiation-inducible promoter, followed by radiation has been demonstrated in a phase I clinical trial of 30 patients with solid tumors, 21 of 30 patients (70%) demonstrated objective tumor response (five complete, nine partial, and seven minimal responses), with only mild toxicities reported as the most common adverse event, including fever (22%), injection site pain (19%) and chills (19%)[15]. A phase I/II non-randomized study enrolled 50 patients for intra-tumoral TNFerade treatment with 5-fluorouracil and radiotherapy for locally advanced pancreatic cancer (27 patients were administered under EUS guidance and 23 patients through the percutaneous route). Their results showed promise with intra-tumoral TNFerade injection, with an overall median survival of 9.9 mo, and median time-to-tumor progression of 3.6 mo, as one patient had complete response, three had partial response, and twelve had stable disease, while 19 patients had progressive disease. Notably, there was a high safety signal in this study, as 40 serious adverse events were recorded, however we were unable to extract whether these adverse events were in the EUS group or in the percutaneous group, as this information was not supplied by the authors[16]. In a randomized phase III multi-institutional study enrolling 304 patients, 187 were treated with standard of care and TNFerade (95 patients under EUS-

guidance and 91 percutaneously) *vs* 117 who received only standard of care therapy. Although the method was shown to be safe, it did not lead to prolonged survival, as the median overall survival was 10 mo in both the 'standard of care and TNFerade' and the 'standard of care alone' groups. Notably in that study, serious related adverse events occurred in 48 patients (25.7%) of the 'standard of care and TNFerade' group, as compared to 20 patients (16.7%) in the 'standard of care' group ($P = 0.13$); however, the serious adverse events were not detailed and the authors did not report whether those adverse events occurred in the EUS or the percutaneous sub-group of the 'standard of care and TNFerade' group[17]. BC-819 is a double-stranded DNA plasmid designated to target the expression of diphtheria-toxin gene under the control of H19 regulatory sequences, and thus have the potential to treat cancer with H19 overexpression. The pharmacokinetics, tolerability and safety and preliminary efficacy of intra-tumoral-injected BC-19 were assessed in a phase 1/2a study of nine patients with unresectable pancreatic adenocarcinoma. The authors reported no increase in tumor size 4 wk after receipt of first treatment, down staging and conversion into resectable cancer in two patients and partial response in three patients after 3 mo. Remarkably, only one spontaneously-resolving asymptomatic lipase elevation considered to be an adverse event, occurred. BC-819 combined with systemic chemotherapy may have additive therapeutic benefit in these patients[18]. Another oncolytic virus is HF10 that enjoys the unique property of being a spontaneous mutation product of herpes simplex virus-1 without artificial modification. It has a high affinity to tumor cells and high replication leading to antitumor immune response[19]. A phase I clinical trial of EUS-guided intra-tumoral injection of HF10 in combination with erlotinib and gemcitabine in 10 patients with unresectable locally-advanced pancreatic cancer reported three partial responses, four stable disease and two progressive diseases in the nine subjects who completed the treatment. However, five patients showed Grade III myelosuppression and two patients developed serious adverse events (perforation of duodenum, hepatic dysfunction), though these events were considered to be unrelated to HF10. Two patients underwent R0 surgical resection after down staging. The median progression-free survival was 6.3 mo and the overall survival 15.5 mo[20]. The effect of the synthetic double stranded RNA oligonucleotide, STNM01, known to selectively inhibit the expression of carbohydrate sulfotransferase-15 (CHST-15)[21], was explored by Nishimura *et al*[22], who injected STNM01 intra-tumorally with EUS-guidance in six patients with unresectable pancreatic cancer. They reported tumor necrosis in biopsy in four patients and significant reduction of CHST15 in two patients, with an overall survival of 15 mo in these two patients, but only 5.7 mo in the other four patients. The authors concluded that EUS-FNI of STNM01 in these patients is safe and feasible[22]. A previous interesting study with 22 patients aimed to assess the effect of CYL-02, a non-viral gene therapy targeted to sensitize pancreatic cells to chemotherapy, reported promising results. Nine patients showed stable disease up to 6 mo following treatment and two of these patients experienced long-term survival, with a median overall survival of 12.6 mo, and without serious adverse events[23].

INTRA-TUMORAL IMPLANTATION

Zorde Khvalevsky *et al*[24] developed a local prolonged siRNA delivery system (Local Drug EluteR, LODER) releasing siRNA against the mutated KRAS (siG12D LODER), enabling siRNA protection from degradation and prolonged periods of intra-tumoral slow release with proved therapeutic efficacy[24]. The tolerability, efficacy and safety of EUS-guided intra-tumoral injection of miniature biodegradable implant siG12D-LODER, releasing a specific silencing RNA against K-RAS mutations in combination with chemotherapy for locally advanced pancreatic cancer patients was shown in a study by Golan *et al*[25]. Their open-label Phase 1/2a study included 15 patients; of the 12 patients analyzed by computed tomography (CT) scans, 10 demonstrated stable disease and two showed partial response. Seven patients had a decrease in tumor marker CA19-9. The median overall survival was 15.12 mo. Serious adverse events were reported in four patients[25].

COMBINATION'S INJECTION

A recent phase 1 study by Lee *et al*[26] evaluating the safety and tolerability of Ad5-yCD/mutTK(SR39)rep-ADP (Ad5-DS), a replication-competent adenovirus-mediated double-suicide gene therapy in combination with gemcitabine, demonstrated the good

tolerability and safety of this combination[26]. Five of their nine patients with inoperable locally-advanced pancreatic cancer treated with the combination of intravenous gemcitabine and EUS-FNI of dendritic cell followed by intravenous infusion of lymphokine-activated killer cells, showed response without treatment-related severe adverse events[27]. Another study evaluated the feasibility, safety and efficacy of EUS-FNI of zoledronate-pulsed dendritic cell combined with intravenous administration of αβT cells and gemcitabine in 15 patients with locally-advanced pancreatic adenocarcinoma. Most of these patients had clinical response and seven had stable disease; the authors concluded that this combination may have a therapeutic benefit. Adverse events were reported in four patients, two of which were related to gemcitabine[28].

EUS-GUIDED ABLATION THERAPIES

Dedicated ablation devices are designed to perform specific ablative procedures in patients with inoperable pancreatic cancer, or who are at high surgical risk or refuse surgery. The procedures include ethanol ablation, thermal ablation including hybrid cryothermal ablation, radiofrequency ablation (RFA), Photodynamic ablation (PDT) and laser ablation[6]. Table 2 shows all studies of EUS-guided ablation therapies.

ETHANOL ABLATION

Ethanol is an attractive ablative agent due to its wide availability, low cost and efficacy. Once injected it causes rapid coagulation necrosis resulting from protein denaturation, cell membrane lysis and vascular occlusion[29]. Its superiority over the percutaneous route resides in its proximity to the pancreas, allowing precise localization and measurement of the lesion with real-time imaging, thus minimizing damage to surrounding normal tissue[30]. To date, we could identify only one study that reported the effect of EUS-guided ethanol injection in pancreatic adenocarcinoma: Facciorusso *et al*[31] evaluated pain management in 123 patients with pancreatic adenocarcinoma, as well as the treatment's effect on overall survival. That study compared the efficacy and safety of EUS-guided tumor ethanol ablation in combination with CPN (65 patients) *vs* CPN alone (58 patients). The combination therapy was shown to be significantly superior to CPN alone in terms of pain relief (P = 0.005) and complete pain response (P = 0.003), with additional survival benefit (8.3 mo *vs* 6.5 mo, respectively). The median duration of pain relief lasted for 18 d (range 13-20) in the combined group, as compared to 10 d (range 7-14) in the CPN group (P = 0.004)[31].

RFA

The high temperature, ranging between 60-100 °C induced by RFA results in irreversible cellular damage, apoptosis and coagulative necrosis[32]. Additionally, it is believed that RFA induces immunomodulatory activity, with anticancer effect[33]. EUS-guided RFA is a minimally invasive, feasible, easy and safe ablative modality that constitutes the ablative modality of choice for several solid tumors[34]. Several small-case series recently assessed this modality in pancreatic cancer. Three feasibility studies were performed in this field; the first was by Song *et al*[35] in which six patients with unresectable pancreatic ductal adenocarcinoma were enrolled to assess feasibility and safety of this modality. This study demonstrated an ablation area within the tumor by contrast- enhanced EUS, with no major side effects (two patients suffered from mild abdominal pain) and with complete technical success[35]. The second study by Crinò *et al*[36] evaluated the technical success, feasibility and safety of EUS-guided RFA in eight patients with pancreatic adenocarcinoma and one patient with renal cell metastasis; they reported feasibility in eight patients, with no major side effects. One- and 30-d' CT demonstrated necrosis of about 30% of the tumor. Three patients reported mild abdominal pain. One of the nine patients was excluded due to a large necrotic portion[36]. The third study, by Scopelliti *et al*[37] enrolled 10 patients with pancreatic ductal adenocarcinoma, and reported success in all patients, with no major adverse events, and with scan-documented area of necrosis within tumor at 30 d post-ablation[37]. A study of 30 patients examined whether SMAD4 status affects post-RFA

Table 2 Human studies reporting endoscopic ultrasound-guided ablation therapies

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation (patients %)	Serious adverse events, *n*
Facciorusso *et al* [31], 2017	Prospective	III (*n* = 50); IV (*n* = 15)	Ethanol ablation	65	100	8.3	90.7 at week 2	0
Song *et al*[35], 2016	Prospective	III (*n* = 4); IV (*n* = 2)	RFA	6	100	-[1]	Not reported	0
Crinò *et al*[36], 2018	Prospective	III (*n* = 8)	RFA	8	100	-[1]	Not reported	0
Scopelliti *et al* [37], 2018	Prospective	III (*n* = 10)	RFA	10	100	-[1]	Not reported	0
Paiella *et al*[38], 2018	Retrospective	Not reported	RFA	30	100	15	Not reported	0
Bang *et al*[39], 2019	Prospective	II (*n* = 2); III (*n* = 3); IV (*n* = 7)	RFA	12	100	Not reported	Significant	0
Arcidiacono *et al* [41], 2012	Prospective	III (*n* = 22)	HCA	22	72.8	6	Not reported	1[2]
DeWitt *et al*[45], 2019	Prospective	III (*n* = 12)	PDA	12	100	11.5	Not reported	0
Di Matteo *et al* [46], 2018	Prospective	III (*n* = 9)	Laser ablation	9	100	7.4	Not reported	0

[1]Feasibility studies not aimed to assess impact on overall survival.
[2]Minor bleeding in duodenal lumen successfully stopped by hemoclips (1 patient). RFA: Radiofrequency ablation; HCA: Hybrid cryothermal ablation; PDA: Photodynamic ablation; EUS: Endoscopic ultrasound.

disease-specific survival in patients with locally advanced pancreatic adenocarcinoma. Results showed that patients with wild-type SMAD4 survived significantly longer than patients with mutant type SMAD4 (22 mo *vs* 12 mo, respectively) with an overall estimated post-RFA disease-specific survival of 15 mo, probably indicating that this gene may help in selecting patients for RFA[38]. Moreover, a recent study by Bang *et al* [39] assessed the role of EUS-guided RFA for pain relief in pancreatic cancer as compared to EUS-guided CPN, and revealed that the EUS-guided RFA was associated with significant improvement in pain associated with pancreatic cancer ($P < 0.05$), in addition to less-severe gastrointestinal symptoms, with better quality of life and emotional functioning[39].

HYBRID CRYOTHERMAL ABLATION

Using a flexible hybrid bipolar cryotherm probe, it is possible to combine radiofrequency with cryotechnology. Cryo is believed to induce a systemic inflammatory response with an antitumor response in addition to the thermal ablation induced by RFA[40]. Only one prospective clinical trial of this type was conducted in 22 patients with locally-advanced pancreatic cancer. Treating them with this hybrid intervention was technically successful in 72.8% of patients, with median post-ablation survival of 6 mo. The few late complications were mainly related to tumor progression, and the single immediate complication of duodenal bleeding was resolved by placing of hemoclips[41]. However, more data are needed to assess this treatment modality.

PDT

PDT is a tumor-specific ablative treatment performed through a combination of photosensitizing drug administration with EUS-guided light irradiation, resulting in cell death by generating oxygen free radicals[42,43]. EUS-guided PDT was first published by Choi *et al*[44], who reported the first preliminary feasibility data for EUS-PDT in patients suffering from locally advanced pancreaticobiliary malignancies. They

enrolled four patients, the first with pancreatic tail carcinoma, the second with distal CBD carcinoma and two patients with carcinoma of the caudate lobe of the liver. The treatment was effective and safe, as it induced a necrotic area of 4 cm^3 without side effects. Notably, disease remained stable for a mean of 5 mo[44]. Recently, a prospective, dose-escalation phase 1 study of 12 patients with locally-advanced pancreatic cancer, treated with EUS-PDT and subsequent gemcitabine therapy 25 d later, showed tumor necrosis in 50% of patients, median progression-free and overall survival were 2.6 and 11.5 mo, respectively. Two patients were operated on, one of them had a complete response and the other one had a residual 2-mm tumor. Notably, there were eight serious adverse events but none related to EUS or EUS-PDT[45]. More data are needed to assess EUS-guided PDT on survival and palliation.

LASER ABLATION

To date, EUS-guided laser ablation has been reported by a single clinical human study that enrolled nine patients with unresectable pancreatic ductal adenocarcinoma who were unresponsive to previous chemotherapy. These patients were treated by laser ablation suing neodymium-yttrium aluminum garnet (Nd:YAG) laser light with different power settings, by flexible fiber, introduced through 22-gauge fine needle aspiration. The coagulative necrotic ablation area was demonstrated by CT scans at 24 h, 7 and 30 d, and was shown to be optimal with power setting of 4 W/1000J with the largest ablation area without adverse events. The median overall survival was 7.4 mo [46]. However, no data regarding palliative effect was reported, thus more data are warranted.

EUS-FMP

Chemoradiation is offered as adjuvant or neoadjuvant to patients with pancreatic adenocarcinoma; however, one of the major challenges with radiation is the proximity of the pancreas to several vital organs. Intensity-modulated radiation therapy was shown to reduce radiation-induced toxicity in these organs in patients with pancreatic and ampullary cancers[47]. Intra-tumoral FMP serves as a landmark enabling accurate radiation targeting of the tumor with minimal harm to neighboring structures. To date, only several feasibility studies were reported addressing safety and technical success, without reporting the effect on overall survival. The first report of EUS-FMP was published in 2006 by Pishvaian *et al*[48] who successfully placed fiducial markers in six of seven pancreatic cancer patients, with no observed complications[48]. After that, several feasibility studies on FMP under EUS-guidance were reported, showing this to be an easy and safe modality with excellent technical success, enabling accurate radiation targeting and without procedure related adverse events in patients with pancreatic cancer[49-54]. As mentioned earlier, to date, the studies on EUS-guided FMP have reported only technical success and adverse events, with no data on survival and palliative benefit, necessitating further studies to assess their therapeutic effect (Table 3).

EUS-GUIDED BRACHYTHERAPY

EUS-guided brachytherapy is defined as the implantation of radioactive seeds near the pancreatic tumorous tissue, followed by exposure of the seeds to steady emissions of gamma rays which lead to localized ablative effect. About two decades ago, Sun *et al* [55] showed that EUS-guided radioactive seeds into pancreatic tissue in a porcine model is a feasible and safe modality for brachytherapy[55]. The favored radioactive seeds in brachytherapy of the rapidly growing pancreatic cancer are iodine-125 due to their long halftime of 59.7 d, which is appropriate in targeting such rapidly-growing tumors. Importantly, the dose rate of these radioactive seeds is low and their penetration depth does not exceed 1.7 cm, thus minimizing radiation exposure and injury to the neighboring organs[56]. Only a few human studies have been conducted with EUS-guided brachytherapy for pancreatic adenocarcinoma. Sun *et al*[57] reported eight patients with locally advanced pancreatic adenocarcinoma who underwent EUS-guided brachytherapy and showed a favorable effect of this modality on pain severity which was ameliorated in four of the eight patients. The pain decrease lasted for 3.5

Table 3 Human studies reporting endoscopic ultrasound-guided fiducial markers placement, -brachytherapy and -celiac plexus neurolysis

Ref.	Study design	Cancer stage	EUS-guided intervention	Patients No.	Technical success (%)	Median overall survival (mo)	Pain palliation (patients %)	Serious adverse events, *n*
Pishvaian *et al* [48], 2006	Prospective	Unresectable	FMP	7	85.7	-[1]	Not reported	0
Choi *et al*[49], 2014	Prospective	Unresectable	FMP	29	100	-[1]	Not reported	1[2]
Varadarajulu *et al*[50], 2010	Prospective	III (*n* = 9)	FMP	9	100	Not reported	Not reported	0
Park *et al*[51], 2010	Prospective	III (*n* = 57)	FMP	57	94	-[1]	Not reported	0
Sanders *et al* [52], 2010	Prospective	III (*n* = 36); Recurrent (*n* = 15)	FMP	51	90	-[1]	Not reported	1[2]
Dávila *et al*[53], 2014	Prospective	II (*n* = 1); III (*n* = 22)	FMP	23	100	-[1]	Not reported	0
Khashab *et al* [54], 2012	Retrospective	III (*n* = 39)	FMP	39	100	-[1]	Not reported	0
Sun *et al*[57], 2012	Prospective	III (*n* = 8)	Brachytherapy	8	100	8.3	50 at week 33	0
Sun *et al*[58], 2006	Prospective	III (*n* = 8); IV (*n* = 7)	Brachytherapy	15	100	10.6	30 at week 42	3[3]
Jin *et al*[59], 2008	Prospective	II (*n* = 4); III (*n* = 10); IV (*n* = 8)	Brachytherapy	22	100	9	81.8 at week 1	0
Sun *et al*[60], 2017	Retrospective	III (*n* = 18); IV (*n* = 24)	Brachytherapy	42	100	9	Not reported	0
Wiersema *et al* [62], 1996	Prospective	Unresectable	CPN	29	100	Not reported	86 at week 2; 84 at week 4; 79 at week 8; 88 at week 12	0
Levy *et al*[64], 2019	Prospective	II (*n* = 2); III (*n* = 27); IV (*n* = 31)	CPN	60	100	10.46	40.4 at week 12	0
Seicean *et al* [65], 2013	Prospective	Unresectable	CPN	32	100	Not reported	75 at week 2	0
Facciorusso *et al* [31], 2017	Prospective	III (*n* = 48); IV (*n* = 10)	CPN	58	100	6.5	70.6 at week 2	0

[1]Feasibility studies not aimed to assess impact on overall survival.
[2]Pancreatitis supportively treated (1 patient).
[3]Pancreatitis complicated with pseudocyst formation (3 patients).
FMP: Fiducial markers placements; CPN: Celiac plexus neurolysis; EUS: Endoscopic ultrasound.

mo and the patients had a median overall survival time of 8.3 mo with no procedure- or treatment-related adverse events[57]. Another study by Sun *et al*[58] reported this treatment modality in 15 patients, with 5 of 15 patients (33.3%) experiencing clinical benefit as assessed by pain reduction and improved Karnofsky performance status score, with a median time-to-achieve clinical benefit of 2.2 mo. Notably, the median overall survival was 10.6 mo, with only three cases of serious complications of pancreatitis complicated with pseudocysts, and no life-threatening adverse events[58]. Similar results were reported by Jin *et al*[59] in 22 patients who showed partial remission in 13.6% of the patients and stable disease in 45.5% during a 4-wk period. Cancer-related pain improved in 18 patients (81.8%) at 1 wk after the intervention, with an estimated median overall survival of 9 mo and no treatment-related adverse events[59]. Finally, the most recent study of this modality performed by Sun *et al*[60] was in 2017 and included 42 patients; once again the research group demonstrated its safety and efficacy, a median overall survival of 9 mo, and no serious adverse events reported[60] (Table 3).

Table 4 Summary of efficacy and safety of endoscopic ultrasound-guided angio-therapy procedures

Procedure	Intra-tumoral injection therapies	Ablation therapies	Fiducial markers placement, brachytherapy and celiac plexus neurolysis
Technical success	High	High	High
Safety (complications)	Uncertain[1]	Minor	Minor
Efficacy			
Survival	Modest	None	None
Palliation	Not reported	Encouraging	High
Mortality	None	None	None

[1]The two studies that had the highest adverse events rate did not state whether they were in the endoscopic ultrasound or in the percutaneous group. See Table 1.

EUS-CPN/B

Abdominal and back pain is a common complaint in pancreatic adenocarcinoma, occurring in about 80% of patients, and it is severe in the majority of patients[61]. Because most patients are diagnosed at an advanced stage, the treatment is mostly palliative, including pain control. The WHO recommends a step-up approach for the control of pancreatic cancer pain, beginning with non-opioid analgesics and progressing to opioid analgesics with increasing dose according to need. Unresponsive patients, those with intolerable side effects, may be candidates for EUS-CPN/B (October 14, 2008. WHO Steering Group on Pain Guidelines). The first description of EUS-CPN/B was by Wiersema *et al*[62] who reported the first human study on EUS-guided brachytherapy in 1996, injection of bupivacaine and 98% dehydrated absolute alcohol in 29 patients with pancreatic adenocarcinoma. Pain score improved in 86%, 84%, 79% and 88% at weeks 2, 4, 8 and 12 post intervention, respectively[62]. Consequently, this therapy rapidly gained popularity as safe and minimally-invasive with the advantage of real-time imaging of blood vessels, compared to the percutaneous route. CPN/B is achieved by alcohol or phenol injection into or around the celiac plexus/ganglion, resulting in its permanent chemical ablation, while CPB is achieved by injecting a corticosteroid in combination with long-acting anesthetic, thus inhibiting pain transmission to the brain[63]. A recent study by Levy *et al*[64] reported the efficacy of EUS-guided CPN/B on the pain score of 60 patients with a pain response rate in 40.4% at 12 wk after intervention, and with an overall survival rate of 10.46 mo[64]. Similarly, another recent study by Facciorusso *et al*[31] reported efficacy in 58 patients, among them 41 patients (70.6%) who achieved pain relief within a median time of 5 d and median pain duration relief of 10 wk, with an overall survival rate of 6.5 mo[31]. The beneficial effect of this modality was shown in a previous study by Seicean *et al*[65] who reported significant pain improvement in 24 (75%) out of 32 patients, and without significant adverse events[65]. Minor side effects of CPN/B including abdominal pain, diarrhea and hypotension due to autonomic nervous system disruption are usually self-limiting. Rare serious adverse events were reported in case reports, including fatal celiac artery thrombosis causing infarction[66], paralysis from anterior spinal cord infection[67] and necrotic gastric perforation[68]. Given the high efficacy of EUS-guided EUS-CPN/B and rarity of adverse events, the latest (1/2020) version of the National Comprehensive Cancer Network (NCCN) guidelines, recommends EUS-CPN for pain palliation in severe pain unresponsive to around-the-clock analgesics or undesirable analgesics side effects[69] (Table 3).

COMBINED EUS AND ERCP IN PANCREATIC ADENOCARCINOMA TREATMENT

Bile duct obstruction with resultant obstructive jaundice and occasional-disabling pruritus is among the most common symptoms of pancreatic head adenocarcinoma. It is usually drained through bile duct stenting introduced *via* ERCP. Employing the beneficial effect of brachytherapy using the radioactive seeds iodine-125, Liu *et al*[70]

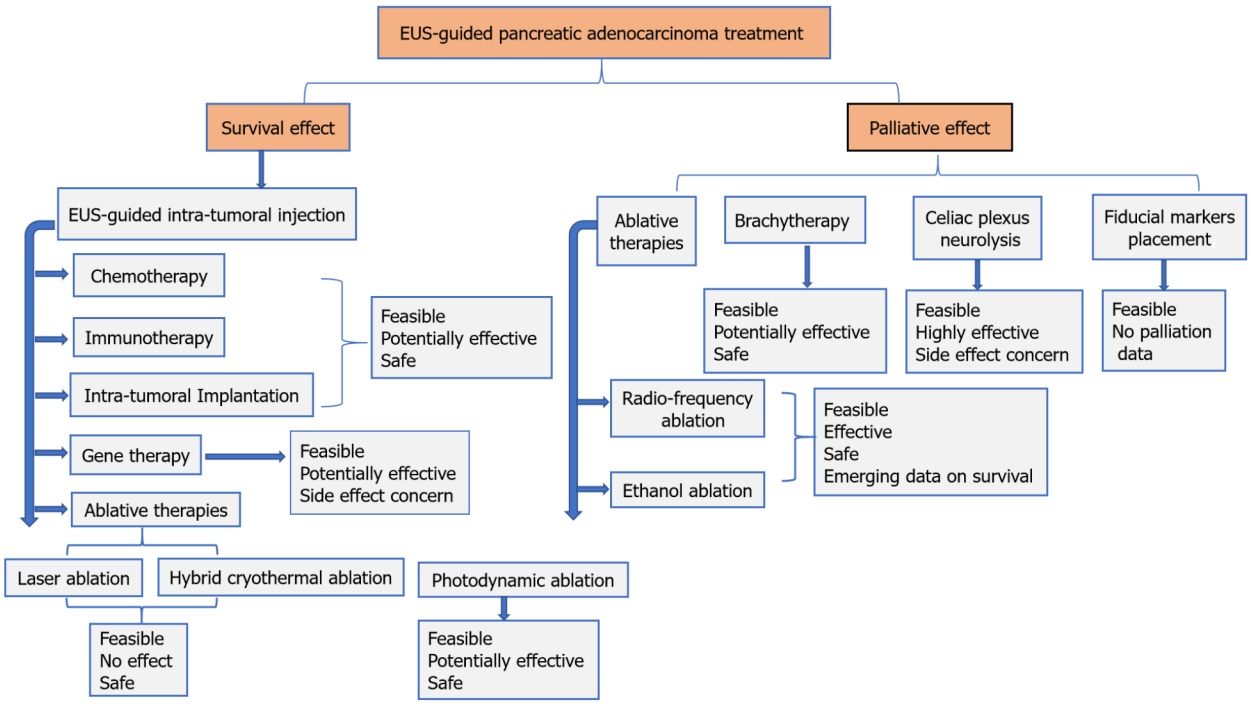


Figure 1 Demonstrates the available endoscopic ultrasound-guided treatment options in pancreatic adenocarcinoma. EUS: Endoscopic ultrasound.

reported that brachytherapy through a preloaded pancreatic stent with iodine-125 seeds, was feasible and safe in an animal experiment using pigs[70]. Two years later, the Liu *et al*[71] group reported the feasibility and tolerability, in a pilot study, of combined radioactive stents with metallic and/or plastic stent in peripancreatic head advanced carcinomas, with stable disease in 72.7% of patients[71]. A recent retrospective study evaluated the role of EUS and/or percutaneous ultrasound-guided iodine-125 seed implantation in 50 patients with unresectable pancreatic carcinoma combined with prior biliary stenting *via* ERCP *vs* biliary stenting alone in 51 patients. They reported longer survival, increased pain reduction with improved life quality, postponed gastric outlet obstruction and longer stent patency in the combination treatment group[72].

SUMMARY

Overall, we identified 12 prospective studies including 261 patients, most in stage III of disease, that utilized EUS-guided intra-tumoral injection therapies and mainly reported effect on patient survival. These studies reported complete technical success without significant effect on overall survival rate, but with several severe adverse events varying in occurrence among the studies. Similarly, in the EUS-guided ablation therapies, we identified one retrospective and eight prospective studies that included 174 patients and reported excellent technical success and minor adverse events, but an inconclusive effect on survival, as half of the studies were feasibility studies not reporting overall survival. Only two of those studies reported pain palliation, however the palliation was significant, thus leading to a hope for performing this treatment for palliative purposes. Finally, we identified seven studies on FMPs, most of them were feasibility studies showing high technical success and minor adverse events. Four studies on brachytherapy included 87 patients and four studies on CPN including 179 patients, with significant improvement in pain ranging from 33% to 90% of patients, no survival benefit and no serious procedure-related adverse events (Table 4).

CONCLUSIONS

In recent years we have witnessed a great advance in interventional and therapeutic EUS. With these developments, EUS has become the preferred alternative for intra-

tumoral injections, ablative therapies, implantation therapies, FMP, brachytherapy, and CPN/B in advanced pancreatic adenocarcinoma. Most EUS-FNI treatments are still far from optimal, and are still in their early stage with little available data, generated from small trials. Figure 1 summarizes the available treatment options for pancreatic adenocarcinoma. The EUS-guided ablation therapies, although encouraging, are far from being standardized. These techniques are in the midst of a long process, necessitating the performance of large prospective randomized controlled studies that compare the different treatment approaches combined with chemo +/- radiotherapy, with respect to success, efficacy, safety and survival. Finally, EUS-guided FMP and brachytherapy are easy, safe and promising modalities, but studies comparing them with the conventional approach of radiotherapy are lacking, while EUS-guided CPN/B is a feasible and accepted tool in pancreatic cancer-related pain control.

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ORIGINAL ARTICLE

Basic Study

Melatonin prevents oxidative stress, inflammatory activity, and DNA damage in cirrhotic rats

Josieli R Colares, Renata M Hartmann, Elizângela G Schemitt, Sandielly R B Fonseca, Marilda S Brasil, Jaqueline N Picada, Alexandre S Dias, Aline F Bueno, Cláudio A Marroni, Norma P Marroni

ORCID number: Josieli R Colares 0000-0002-0482-204X; Renata M Hartmann 0000-0001-8667-517X; Elizângela G Schemitt 0000-0003-3015-0192; Sandielly R B Fonseca 0000-0003-2662-5116; Marilda S Brasil 0000-0002-8420-3045; Jaqueline N Picada 0000-0003-2360-1557; Alexandre S Dias 0000-0002-0609-4779; Aline F Bueno 0000-0001-9382-5914; Cláudio A Marroni 0000-0002-1718-6548; Norma P Marroni 0000-0001-7856-7953.

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Institutional review board statement: This study was conducted at the Laboratory of Experimental Hepatology and Gastroenterology, Hospital de Clínicas of Porto Alegre after approval from the Institutional Animal Care and Use Committee (opinion no. CEUA-HCPA No. 2016-0373).

Josieli R Colares, Renata M Hartmann, Elizângela G Schemitt, Sandielly R B Fonseca, Norma P Marroni, Medical Sciences Program, Universidade Federal do Rio Grande do Sul, Porto Alegre 90050-170, Brazil

Marilda S Brasil, Norma P Marroni, Biological Sciences Program, Universidade Federal do Rio Grande do Sul, Porto Alegre 90050-170, Brazil

Jaqueline N Picada, Cellular and Molecular Biology Program, Lutheran University of Brazil (ULBRA), Canoas 92425-900, Brazil

Alexandre S Dias, Aline F Bueno, Pneumological Sciences Program, Universidade Federal do Rio Grande do Sul, Porto Alegre 90050-170, Brazil

Cláudio A Marroni, Posgraduate Program in Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre 90050-170, Brazil

Corresponding author: Norma P Marroni, PhD, Doctor, Full Professor, Research Scientist, Medical Sciences Program, Universidade Federal do Rio Grande do Sul, Rua Jose Kanan Aranha, Porto Alegre 90050-170, Brazil. nmarroni@terra.com.br

Abstract

BACKGROUND

Cirrhosis is an important health problem characterized by a significant change in liver parenchyma. In animals, this can be reproduced by an experimental model of bile duct ligation (BDL). Melatonin (MLT) is a physiological hormone synthesized from serotonin that has been studied for its beneficial properties, including its antioxidant potential.

AIM

To evaluate MLT's effects on oxidative stress, the inflammatory process, and DNA damage in an experimental model of secondary biliary cirrhosis.

METHODS

Male Wistar rats were divided into 4 groups: Control (CO), CO + MLT, BDL, and BDL + MLT. MLT was administered (20 mg/kg) daily beginning on day 15 after biliary obstruction. On day 29 the animals were killed. Blood samples, liver tissue, and bone marrow were collected for further analysis.

Institutional animal care and use committee statement: This study was conducted at the Animal Experimentation Unit, Hospital de Clínicas of Porto Alegre after approval from the Institutional Animal Care and Use Committee (opinion no. CEUA-HCPA No. 2016-0373).

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RESULTS

BDL caused changes in biochemical and histological parameters and markers of inflammatory process. Thiobarbituric acid (0.46 ± 0.01) reactive substance levels, superoxide dismutase activity (2.30 ± 0.07) and nitric oxide levels (2.48 ± 0.36) were significantly lower ($P < 0.001$) n the groups that received MLT. DNA damage was also lower ($P < 0.001$) in MLT-treated groups (171.6 ± 32.9) than the BDL-only group (295.5 ± 34.8). Tissue damage and the expression of nuclear factor kappa B, interleukin-1β, Nrf2, NQO1 and Hsp70 were significantly lower in animals treated with MLT ($P < 0.001$).

CONCLUSION

When administered to rats with BDL-induced secondary biliary cirrhosis, MLT effectively restored the evaluated parameters.

Key Words: Antioxidants; Secondary biliary cirrhosis; Oxidative stress; Melatonin; Bile duct ligation

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Core Tip: The experimental model of secondary biliary cirrhosis, by ligation of the main bile duct, mimics the clinical situation, with biochemical, enzymatic, histological changes, and similar biological, inflammatory, genotoxic markers and oxidative stress triggers. Melatonin, used as an antioxidant therapeutic agent, has been shown to be effective in reversing the changes caused at different levels due to its antioxidant, anti-inflammatory, cytoprotective action, including a reduction in DNA damage, with significant improvement and future therapeutic potential.

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INTRODUCTION

Secondary biliary cirrhosis, a late complication of prolonged obstruction of the extrahepatic bile duct, causes cholestasis[1]. Cholestatic liver damage, defined according to histopathological and biochemical criteria as an accumulation of toxic bile acids, plays a fundamental role in liver necrosis and fibrosis[2-4].

Prolonged common bile duct obstruction in rats is an experimental model that induces secondary biliary cirrhosis in 28 d and mimics human liver disease in clinical, laboratory and histological parameters[2,3,5].

Oxidative stress plays a determinant role in the pathophysiology of liver diseases due to the accumulation of reactive oxygen species, protein oxidation, lipid peroxidation (LPO) and DNA damage, which has also been evaluated in different experimental models[3,5-7].

Oxidative stress, due to the accumulation of reactive oxygen species, destabilizes cell homeostasis. Nrf2, which regulates cellular response to oxidative damage and the expression of most antioxidant enzymes under normal conditions, is kept inactive by the protein Keap1. In stressful situations for the cell, Nrf2 dissociates and translocates to the nucleus, where it binds to the promoter sequence as an antioxidant response element and activates genes, initiating the transcription of new antioxidant enzymes[8-10]. The Keap1/Nrf2 pathway is responsible for regulating both cytoprotective genes and defense antioxidants, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as glutathione reductase, gamma glutamylcysteine ligase, xenobiotic detoxification, NAD (P) H: Quinone oxidoreductase 1 (NQO1), and genes from the glutathione S-transferase family[11,12].

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Increased production of reactive oxygen species, the presence of inflammatory mediators such as interleukins (IL-1β and IL-6), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-kB), and increased nitric oxide levels may be related to the development of fibrosis in liver cirrhosis[2,3].

Hsp70 is an endogenous protein that plays a protective role in cell function, assisting in protein synthesis. Studies have shown that Hsp70 induction occurs in response to various stimuli, such as exposure to toxins, glucose deprivation, and reactive oxygen species formation, as well as liver cirrhosis[13,14].

Melatonin (MLT), N-acetyl-5-methoxytryptamine, is a hormone synthesized by the pineal gland, which is produced rhythmically and is inhibited by light[15,16]. The antioxidant effect of MLT is related to its amphiphilic chemical structure, which facilitates its crossing through biological barriers and allows activity in both aqueous and lipid environments[15,17]. The numerous attributes of MLT include antioxidant capacity and anti-inflammatory and immunomodulatory effects[18]. Exogenous MLT has protective effects on hepatic ischemia-reperfusion injury[19]. The inadequate expression of MLT predisposes liver cells to immune- and oxidative stress-related damage. MLT, *via* epigenetic modulation, was able to suppress NF-κB signaling activation and protecting against apoptotic signaling induced by either oxidative stress or high concentrations of bile[20]. MLT, participates in regulating multiple physiological functions, including sleep, circadian rhythms, and neuroendocrine processes.

Current evidence shows that MT protects against liver injury by inhibiting oxidation, inflammation, haematopoietic stem cell (HSC) proliferation, and hepatocyte apoptosis, thereby inhibiting the progression of liver cirrhosis[17].

Inflammation and oxidative stress play an important role in the pathophysiology of cirrhosis and other liver diseases, which is why pharmacological interventions can change the evolution of the disease. Non-alcoholic fatty liver disease (NAFLD) patients who underwent treatment with Essentiale Forte and tryptophan or MT for 14 mo had reduced expression of GGTP, triglycerides, low-density lipoprotein cholesterol and proinflammatory cytokines including IL-1, IL-6 and TNF-α, although there was no significant difference in alanine aminotransferase level or other biochemical parameters[21]. NAFLD patients treated with MT were found to have significantly lower aspartate aminotransferase (AST) and high-sensitivity C-reactive protein levels and a better liver grade than those who received placebo[22]. MLT seems safe and effective in the short term as a sedative in patients with CTP classes A and B cirrhosis and SD. This finding may have clinical applications in the holistic management of patients with cirrhosis[23]. There is a positive association between high serum MLT levels prior to LT, one-year survival after LT, and total antioxidant capacity[24].

The aim of the present study was to evaluate MLT's effects on oxidative stress, the inflammatory process, and DNA damage in an experimental model of secondary biliary cirrhosis.

MATERIALS AND METHODS

Ethical considerations

This study was conducted at the Animal Experimentation Unit and the Second Laboratory of Experimental and Inflammatory Pneumological Sciences of the Hospital de Clínicas de Porto Alegre after approval by the Institutional Commission for the Treatment and Use of Animals (protocol 2016-0373).

Animal handling was carried out according to Brazilian federal legislation (Law 11794/2008), Brazilian Council for the Control of Animal Experimentation (CONCEA) rules, the State Code for the Protection of Animals, and local legislation regarding the care and use of animals in experimental research.

Experimental procedures

Twenty-four male Wistar rats (mean weight 300 g) were divided into four experimental groups: Control (CO), control treated with MLT (CO + MLT), bile duct ligation (BDL) and BDL treated with MLT (BDL + MLT). During the experiment, the animals were maintained in cages (47 cm × 34 cm × 18 cm) lined with wood shavings, under a 12 h light/dark cycle and controlled temperature (18-22 °C), with free access to food and water.

On the first day of the experiment, BDL surgery was performed, as well as simulated surgery in the CO and CO + MLT groups according to Kountouras *et al* (1984)[25]. On the 15th day of the experiment, the animals began receiving MLT in daily

doses of 20 mg/kg of body weight. The treatment continued until the 28th day.

On the 29th day, the animals were weighed and anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (95 mg/kg) and 2% xylazine hydrochloride (8 mg/kg). Blood was then collected from the retro-orbital plexus with a glass capillary tube and placed in a test tube with heparin to prevent coagulation.

After blood collection, the animals were sacrificed by anesthetic overdose (three times the therapeutic dose, according to the CONCEA guidelines. Upon confirmation of death, a ventral midline laparotomy was performed, after abdominal trichotomy and disinfection. The liver was removed, sectioned, and stored for subsequent analysis. One liver fragment was submerged in a 10% formaldehyde solution for 24 h for histological examination, one fragment was stored in a fixative containing glutaraldehyde for subsequent analysis by scanning electron microscopy, a third fragment was frozen at -80 °C for further analysis, and bone marrow samples were collected for the micronucleus test.

Histological analysis of hepatic tissue

After dissection, the liver was placed in 10% buffered formalin and later embedded in paraffin blocks. The paraffin blocks were then attached to a microtome (Leitz-1512 Microtome, Leitz, Wetzlar, Germany) for cutting. The slides were then stained with hematoxylin-eosin and washed in running water. In the dehydration phase, the structures went through a series of three baths: One in absolute alcohol and two in xylol. The cover slip was then fixed into place using Canada balsam or Entellan, which completed the preparation process. The slides were analyzed with a Nikon Labophot binocular microscope equipped with a digital camera. Using the Image-Plus software (Media Cybernetics, Bethesda, MD, United States), images were captured at different magnifications.

Microscopy evaluation

Tissue samples were fixed in 10% formalin and embedded in paraffin. The paraffin blocks were then attached to a microtome (Leitz® 1512) and cut in 3 μm sections. The slides were stained in hematoxylin-eosin for 5 min each and then washed in running water. In the dehydration phase, the structures went through a series of three baths: One in absolute alcohol and 2 in xylol. The cover slip was fixed into place using Canada Balsam. The slides were analyzed with a microscope equipped with a digital camera. Using Image-Plus software, images were captured at 200× magnification.

Preparation of homogenates

Nine ml of phosphate buffer were added *per* gram of tissue, which was then homogenized in an Ultra-Turrax homogenizer (IKA-Werk, Staufen, Germany) for approximately 40 s and kept on ice, followed by centrifugation in a SORVALL RC-5B refrigerated Superspeed Centrifuge (Du Pont Instruments, Miami, FL, United States) for 10 min at 4000 rpm[26]. The precipitate was discarded and the supernatant was used to quantify the proteins.

Microscopic analysis of liver tissue

Liver samples were collected for scanning electron microscopy. After collection, the samples were immersed in a fixative solution containing glutaraldehyde. The samples were then washed, dehydrated, desiccated and metallized, followed by analysis in an electron microscope (Jeol JSM-T330, Tokyo, Japan) at 5000× magnification.

Analysis of biochemical and spectrophotometric parameters

Liver integrity was assessed by measuring liver enzymes AST, alanine aminotransferase, and alkaline phosphatase in plasma with a Liquiform Labtest® kit (a kinetic spectrophotometric assay).

The protein content in liver homogenate was determined using the Bradford method[27]. LPO was investigated with a thiobarbituric acid reactive substances (TBARS) assay, with the concentration expressed in nmol/mg of protein[28]. SOD activity was measured in a plate reader, evaluating its ability to inhibit the superoxide radical from reacting with adrenaline. The results were expressed in SOD units *per* milligram of protein[29]. The production of nitric oxide metabolites [nitrites (NO_2) /nitrates(NO_3)] was measured indirectly with the Griess reaction. This assay is based on the enzymatic reduction of NO_3 to NO_2 in the presence of nitrate reductase and NQO1, with subsequent colorimetric determination of NO_2 using the Griess reagent (a mixture of sulfanilamide and NO_2-specific *N*-[1-Naphthyl]ethylenediamine). The results were expressed in mmol/L[30].

DNA damage analysis

To assess DNA damage, we used the alkaline version of the comet assay described by Tice *et al* (2000)[31]. Aliquots of 10 ml of liver cell suspension were mixed with 0.75% low-melting agarose and placed 1.5% agarose-coated slides; these slides were immersed in a lysis solution, which allowed the migration of DNA fragments by electrophoresis. The results were expressed in a damage index, obtained by visual assessment of damage classes (from 0 to 4), and damage frequency, calculated from the number of cells with *vs* without tails[32].

As described by Mavournin *et al* (1990)[33], bone marrow samples were collected from both femurs for the micronucleus test. To collect the samples, the proximal end of each femur was cut to expose the spinal canal, allowing extraction. To count normochromatic erythrocytes, polychromatic erythrocytes (PCE), and micronuclei in the PCE, an optical microscope with an immersion objective was used, and at least 2000 PCE were analyzed *per* animal. The polychromatic/normochromatic erythrocyte ratio was also determined by assessing the frequency of PCE in 1000 erythrocytes from each animal[34].

Multiplex analysis

IL-1β cytokine levels were assessed using a microsphere-based multiplex assay (MILLIPLEX Map Kit, Rat Cytokine/Chemokine Magnetic Bead Panel, Cat. Nº. RECYTMAG-65K; Millipore Corporation, Billerica, MA, United States). Cytokine detection was performed by adding specific fluorescence conjugated antibodies.

Quantification was based on a standard curve with known dilutions, and the results were expressed in pg/mg. The samples were analyzed in a Luminex 200TM reader (Luminex, Austin, TX, United States) according to manufacturer instructions.

Immunohistochemical analysis

The slides, pre-incubated with 10% rabbit serum at room temperature to block possible unwanted reactions from the secondary antibody, were incubated with monoclonal antibodies (Nrf2, NQO1, NF-κB and Hsp70) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) overnight at 4 ºC, followed by incubation with a secondary antibody for one hour at room temperature. After 60 min at room temperature, they were treated with EnVision reagent and washed three times with phosphate-buffered saline. The nuclei were counterstained with hematoxylin. The primary antibody was diluted in phosphate-buffered saline, which contained bovine albumin as a negative control. The results were evaluated by blinded pathologists using a microscope equipped with a digital camera and Image-Plus software (Media Cybernetics).

Western blot analysis

Cytoplasmic and nuclear extracts were prepared from liver homogenates using a specific lysis buffer and protease inhibitors[35]. The supernatant fraction was collected and stored in aliquots at -80 °C for further analysis. The lysed proteins were separated by dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% skim milk in Tris buffer containing 0.05% Tween 20 (TTBS) for 60 min at 37 °C. Thereafter, the primary antibodies were incubated and stirred overnight at 4 ºC. The following proteins were evaluated: NF-kB (65 kDa and Hsp70; 70 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, United States) diluted from 1:200 to 1:1000 with Tris-buffered saline in skim milk at 5%. HRP-antibody protein biomarker detection was performed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The density of specific bands was quantified through image densitometry software (Scion Image, Frederick, MD, United States)[36,37].

Statistical analysis

The quantitative data were expressed as mean ± standard error. The groups were compared using unilateral analysis of variance. The Student-Newman-Keuls procedure was used to find differences in means (SPSS, version 17.0). The Tukey test was used for the comet assay. The data were analyzed in GraphPad InsTat 3.1, with $P < 0.05$ considered significant.

RESULTS

Histological analysis

In the histological analysis, hematoxylin-eosin staining in the BDL group revealed changes in the liver parenchyma, a loss of hepatocyte cords, and the presence of inflammatory infiltrate (Figure 1, black arrows). In the BDL + MLT group, we observed restructuring of these changes, including the formation of hepatocyte cords, decreased inflammatory infiltrate, and preserved hepatocytes (Figure 1). In the CO and CO + MLT groups, the liver parenchyma was unchanged.

Scanning electron microscopy analysis

According to scanning electron microscopy of the liver samples, the ciliated membrane, which covers hepatocytes involved in inflammatory process signaling in response to damage, was intact in the CO and the CO + MLT groups. This membrane was damaged in the BDL group, although in the BDL + MLT group the membrane had been restructured (Figure 2).

Analysis of biochemical and spectrophotometric parameters

Liver enzyme alterations: All plasma liver enzymes in the BDL group were significantly higher than in the control groups (CO and CO + MLT), and these values were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Figure 3).

Protein evaluation, LPO, antioxidant enzyme SOD and nitric oxide: The total protein levels (Figure 4A) in liver homogenate were significantly higher in the BDL group than the control groups ($P < 0.001$) and were significantly higher in the BDL + MLT group than the BDL group ($P < 0.001$).

The LPO level was significantly higher in the BDL group than the CO and CO + MLT groups and was significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

There was significantly less SOD activity in the BDL group than the control groups (CO and CO + MLT) and significantly more activity in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

The levels of nitric oxide metabolites (nitrites and nitrates) were significantly higher in the BDL group than the CO and CO + MLT groups, but they were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 1).

DNA damage analysis

In the comet assay analysis, the BDL group had a significantly higher damage index and damage frequency than the CO and CO + MLT groups. These parameters were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 2).

The micronucleus frequency was significantly higher in the BDL group than the CO and CO + MLT groups and was significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$) (Table 3). No significant differences were found between the groups in the polychromatic/normochromatic erythrocyte ratio, which indicated no toxicity in the bone marrow.

Multiplex analysis

The pro-inflammatory cytokine IL-1β levels were significantly higher in the BDL group than the CO and CO + MLT groups ($P < 0.001$) and were significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$), as can be seen in Figure 4B.

Immunohistochemistry and quantification of Nrf2, NF-kB, Hsp70 and NQO1

We observed significantly higher expression of NF-kB (Figure 5A) and Hsp70 (Figure 6A) in the BDL group than the control groups and significantly lower expression in the BDL + MLT group than the BDL group ($P < 0.001$).

There was significantly lower expression of Nrf2 in the BDL group than in the CO and CO + MLT groups but significantly higher expression in the BDL + MLT group than the BDL group (Figure 7). There was significantly higher expression of NQO1 in the BDL group than the CO and CO + MLT groups but significantly lower expression in the BDL + MLT group than the BDL group ($P < 0.001$)(Figure 8).

Expression of NF-kB and Hsp70

Western blot analysis of NF-kB (Figure 5B) and Hsp70 (Figure 6B) expression showed that they were significantly higher in the BDL group than the control groups (CO and

Table 1 Activity of lipid peroxidation levels, antioxidant enzyme superoxide dismutase, and nitric oxide levels in the experimental groups

Groups	TBARS	SOD	NO_2/NO_3
CO	0.29 ± 0.01	2.62 ± 0.11	1.69 ± 0.08
CO + MLT	0.34 ± 0.02	2.43 ± 0.05	1.76 ± 0.09
BDL	3.43 ± 0.08[a]	0.61 ± 0.05[a]	5.53 ± 0.30[a]
BDL + MLT	0.46 ± 0.01[b]	2.30 ± 0.07[b]	2.48 ± 0.36[b]

[a]$P < 0.001$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups.
[b]$P < 0.001$: Significant reduction in the BDL + MLT group in relation to the BDL group.
CO: Control; MLT: Melatonin; BDL: Bile duct ligation; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase.

Table 2 Comet assay in liver tissue of rats that underwent bile duct ligation treated or not with melatonin

Group	Damage index	Damage frequency
CO	77.4 ± 8.6	72.2 ± 7.3
CO + MLT	72.6 ± 16.2	67.8 ± 14.8
BDL	295.5 ± 34.8[a]	100.0 ± 0.0[c]
BDL + MLT	171.6 ± 32.9[b]	91.6 ± 9.5

The damage index can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage 100 × 4). The damage frequency was calculated based on the number of cells with *vs* without tails.
[a]$P < 0.001$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups.
[b]$P < 0.001$: Significant reduction in the BDL + MLT group in relation to the BDL group.
[c]$P < 0.01$: Significant increase in the BDL group in relation to the CO and CO + MLT groups.
CO: Control; MLT: Melatonin; BDL: Bile duct ligation; TBARS: Thiobarbituric acid reactive substances.

Table 3 Micronucleus test in the bone marrow of the rats that underwent bile duct ligation, treated or not with melatonin

Groups	MNPCE[1] in 2000 PCE	Ratio PCE/NCE[2]
CO	4.0 ± 1.5	1.1 ± 0.2
CO + MLT	3.3 ± 1.0	1.1 ± 0.4
BDL	7.8 ± 1.3[a]	1.0 ± 0.6
BDL + MLT	6.8 ± 1.9	0.9 ± 0.5

[1]MNPCE: Micronuclei in polychromatic erythrocytes.
[2]PCE/NCE ratio: Proportion of polychromatic erythrocytes/normochromatic erythrocytes.
[a]$P < 0.01$: Significant increase in the bile duct ligation group in relation to the control and CO + melatonin groups. (Analysis of variance, Tukey test).
CO: Control; MLT: Melatonin; BDL: Bile duct ligation.

CO + MLT) and significantly lower in the BDL + MLT group than the BDL group ($P < 0.001$).

DISCUSSION

Common bile duct obstruction causes hepatocellular damage and an inflammatory response due to the accumulation of bile salts in the liver, which promotes cytokine production, hepatocellular injury, and the healing process, leading to an accumulation of collagen, fibrosis, and liver cirrhosis[38]. The BDL model has been used to study the numerous molecular signaling pathways involved in secondary biliary cirrhosis[3].

MLT has shown protective effects in different models, inhibiting oxidative stress, inflammatory signaling, autophagy, hepatocyte apoptosis, cell and tissue damage[3,16,

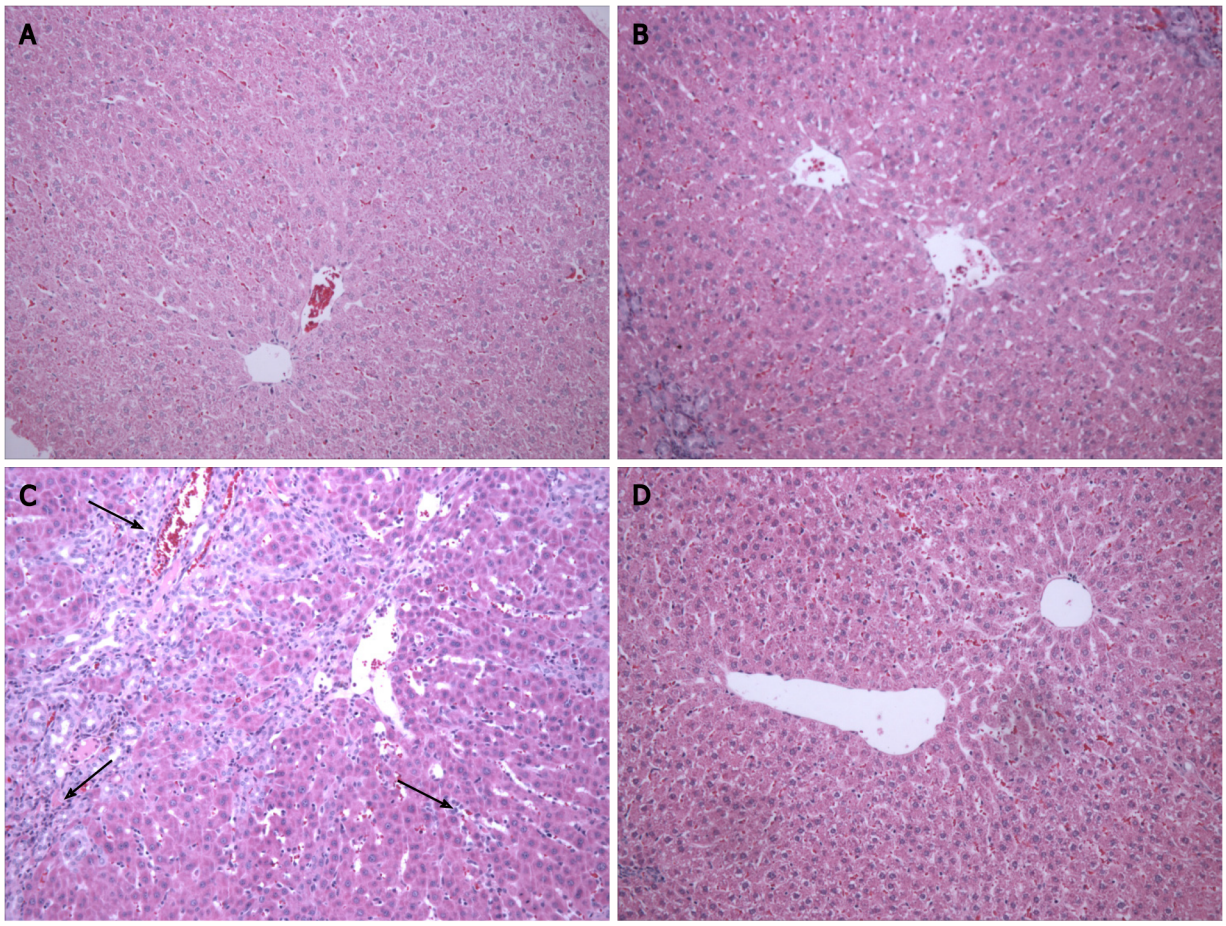


Figure 1 Photomicrograph of hepatic tissue at 200 × magnification in the different experimental groups. The control (CO) and CO + melatonin (MLT) groups had normal liver parenchyma. There was inflammatory infiltrate (black arrows) and a change in the parenchyma in the bile duct ligation (BDL) group. Parenchymal restructuring occurred in the BDL + MLT group. A: CO; B: CO + MLT; C: BDL; D: BDL + MLT.

17,39-41], and it seems safe and effective in the short term as a sedative in patients with CTP classes A and B cirrhosis and SD. This finding may have clinical applications in the holistic management of patients with cirrhosis[23].

The present study investigated the effects of MLT on oxidative, inflammatory, tissue, and cellular injury in an experimental model of secondary biliary cirrhosis. It was found that a dose of 20 mg/kg of MLT, which has already been investigated in other studies by our group, was effective in reducing or modulating oxidative stress, inflammatory processes, and DNA damage. The animals that underwent BDL surgery had a higher expression of the enzymes AST, alanine aminotransferase and alkaline phosphatase than the other groups, indicating possible damage to hepatocyte membranes. When MLT was administered to these animals, we observed a significant decrease in the expression of these enzymes, possibly due to hepatocyte membrane restructuring and reduced liver damage. This corroborates the results of Wu *et al*[12] in a model of BDL and CCL4-induced hepatic fibrosis. These authors found a significant increase in AST and alanine aminotransferase expression, as well as an equally significant reduction in expression after treatment with the antioxidant quercetin. In a model of severe acute liver failure, Schemitt *et al*[42] found an increase in AST, alanine aminotransferase and alkaline phosphatase expression, indicating a loss of liver integrity; high serum levels of these enzymes were related to cell damage and liver cell necrosis, which was reversed with glutamine[42].

Changes in the hepatic parenchyma, including the formation of fibrotic septa and necrosis, are often associated with the process of cirrhosis[12]. Hematoxylin-eosin staining showed disorganized liver tissue in the BDL group, including a loss of hepatocyte cords and the presence of inflammatory infiltrate and fibrosis. However, tissue restructuring had occurred in the BDL + MLT group, and organized tissue was observed in the CO and CO + MLT groups. Scanning electron microscopy revealed that in the control groups (CO and CO + MLT) the ciliated membrane of hepatocytes involved in inflammatory process signaling was intact. In the BDL group, however,

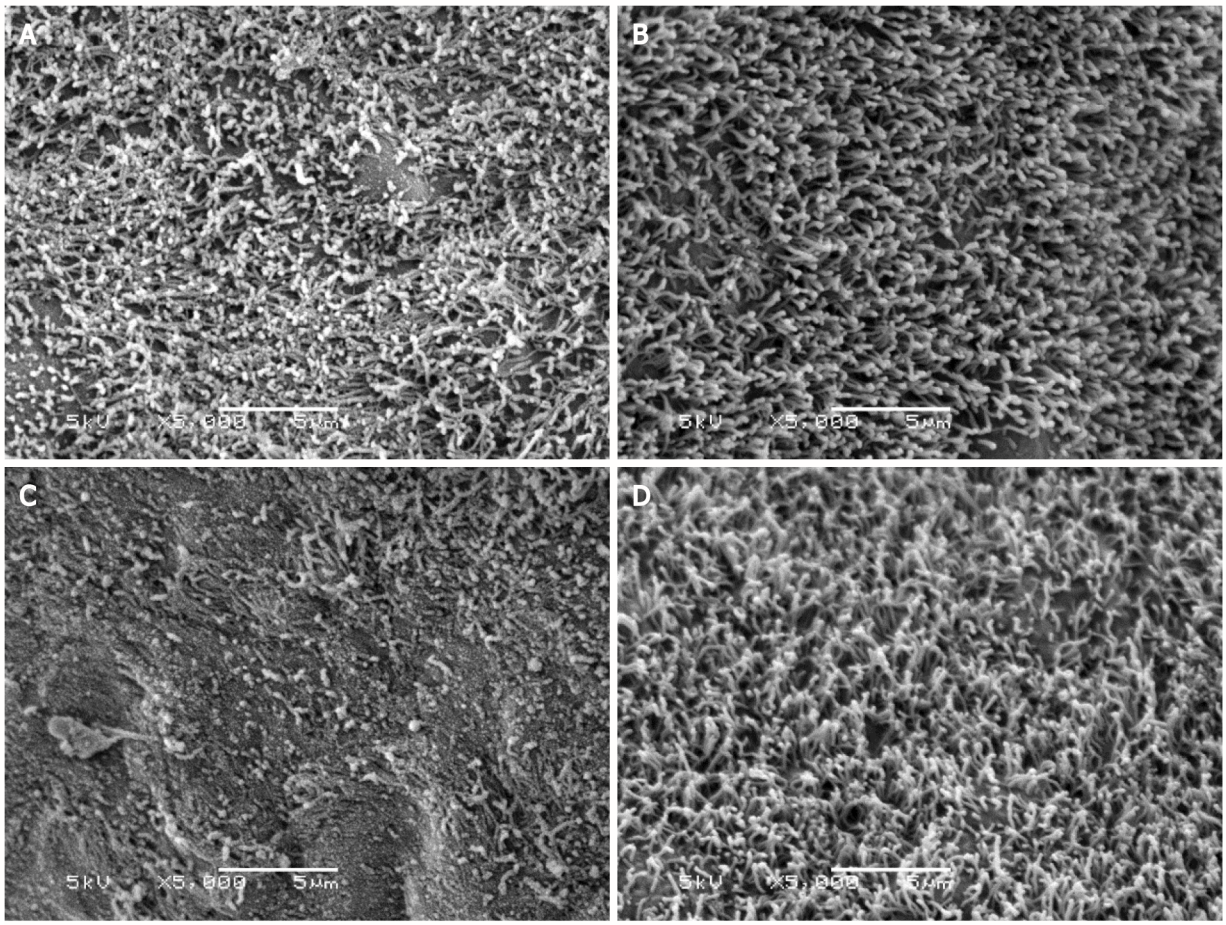


Figure 2 Morphological analysis by scanning electron microscopy of the liver of animals that underwent bile duct ligation surgery. The control (CO) and CO + melatonin (MLT) groups showed an intact ciliated membrane covering the hepatocytes. This membrane is impaired in the bile duct ligation (BDL) group. In contrast, membrane restructuring was observed in the BDL + MLT group. A: CO; B: CO + MLT; C: BDL; D: BDL + MLT.

this membrane was damaged, and in the BDL + MLT group it had been restructured (Figure 2).

In 2016, Wree and Marra[43] described the surface of hepatocytes and their association with reduced cell permeability, as well as a consequent increase in mediators involved in the fibrotic and inflammatory process, which was associated with changes in the ciliated membrane and inflammasome. According to Gonzalez-Navajas[44], inflammation is involved in the pathogenesis of many liver diseases, including cirrhosis, in which many inflammatory cytokines are produced after activation of a multiprotein complex known as inflammasome. However, the origin and mechanisms of hepatic damage mediated by inflammasome are little known[43]. Most acute or chronic liver diseases are accompanied by inflammation, a complex process in response to liver aggression, which causes serious damage to the liver parenchyma[45-47].

In 2017, Giusto *et al*[48] showed that mice with cirrhosis induced by BDL and CCL4 had fibrotic nodules and cellular changes. In 2013, Mazo *et al*[4] observed that in rats submitted in experimental nonalcoholic steatohepatitis, including fibrosis, the administration of N-acetylcysteine restored liver parenchyma.

Studies report that in the pathophysiology of biliary cirrhosis, liver damage is maximized by the action of free radicals. LPO causes the disorganization of cell membranes, resulting in increased membrane permeability and consequent enzyme leakage, leading to cell death. Studies show that plasma malondialdehyde levels may be associated with increased LPO[3,5,42].

In our study, LPO was analyzed with a TBARS assay, and it was significantly higher in the cirrhotic group (BDL) than the other groups, which may be associated with damage to cell membranes. LPO was significantly lower in the BDL + MLT group than the BDL group, which suggests that MLT plays a protective role. These data corroborate a study by Zhu *et al*[49], who, in rats with hepatopulmonary syndrome-BDL induced, observed higher LPO in the cirrhotic group and that Tea polyphenols

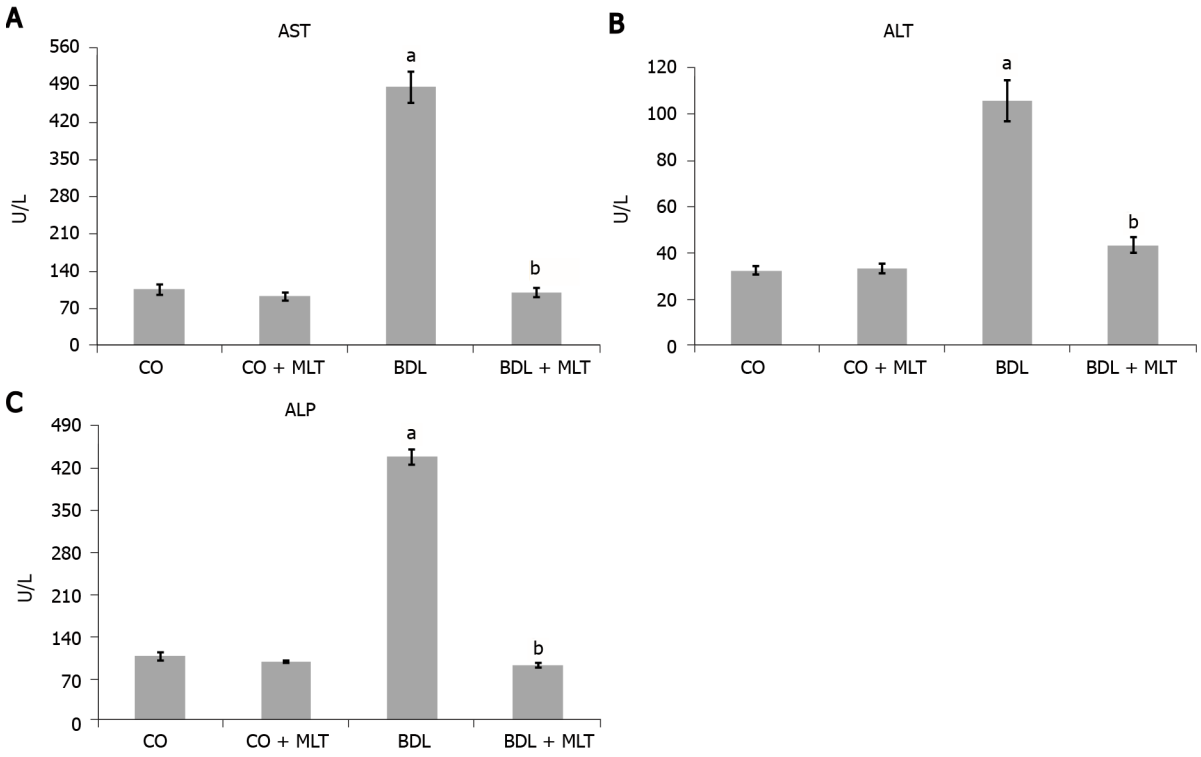


Figure 3 The effect of melatonin on the activity of liver enzymes aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase (alkaline phosphatase) in the plasma of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant increase in relation to the control (CO) and the CO + melatonin (MLT) groups ($^aP < 0.001$). Significant decrease in the bile duct ligation (BDL) group in relation to the BDL + MLT group ($^bP < 0.001$). A: AST; B: ALT; C: ALP.

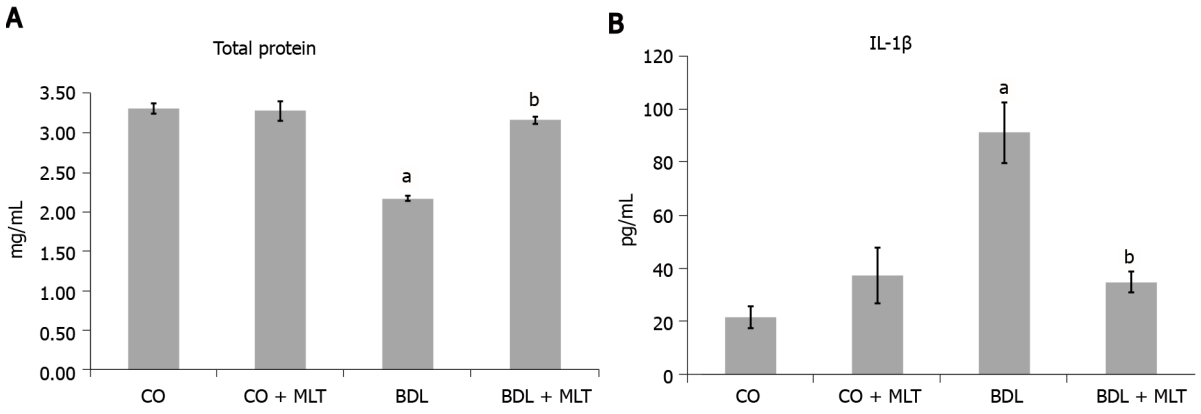


Figure 4 The effect of melatonin on total protein levels and interleukin IL-1β levels in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant decrease/increase in the bile duct ligation (BDL) group in relation to the control (CO) and the CO + melatonin (MLT) groups ($^aP < 0.001$). Significant increase in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$). A: Total protein; B: IL-1β.

significantly decreased LPO. Similar data were observed in other experimental models that administered MLT[3,41,42].

Other authors have observed LPO in the lungs of animals with secondary biliary cirrhosis due to BDL, considering it characteristic of the damage to cell membranes in this experimental model[3,49].

In addition to the lipid damage, which is assessed by increased LPO, reactive oxygen species can damage DNA. Oxidative damage to DNA is common, being the main cause of genomic instability[31]. We observed a significant increase in liver DNA damage in the BDL group, as well as in the frequency of micronuclei in the bone marrow, which suggests increased genomic instability. The significantly better micronucleus and comet assay results in the MLT-treated group suggests that MLT prevented cytogenetic damage, as well as strand breakage and DNA base oxidation in

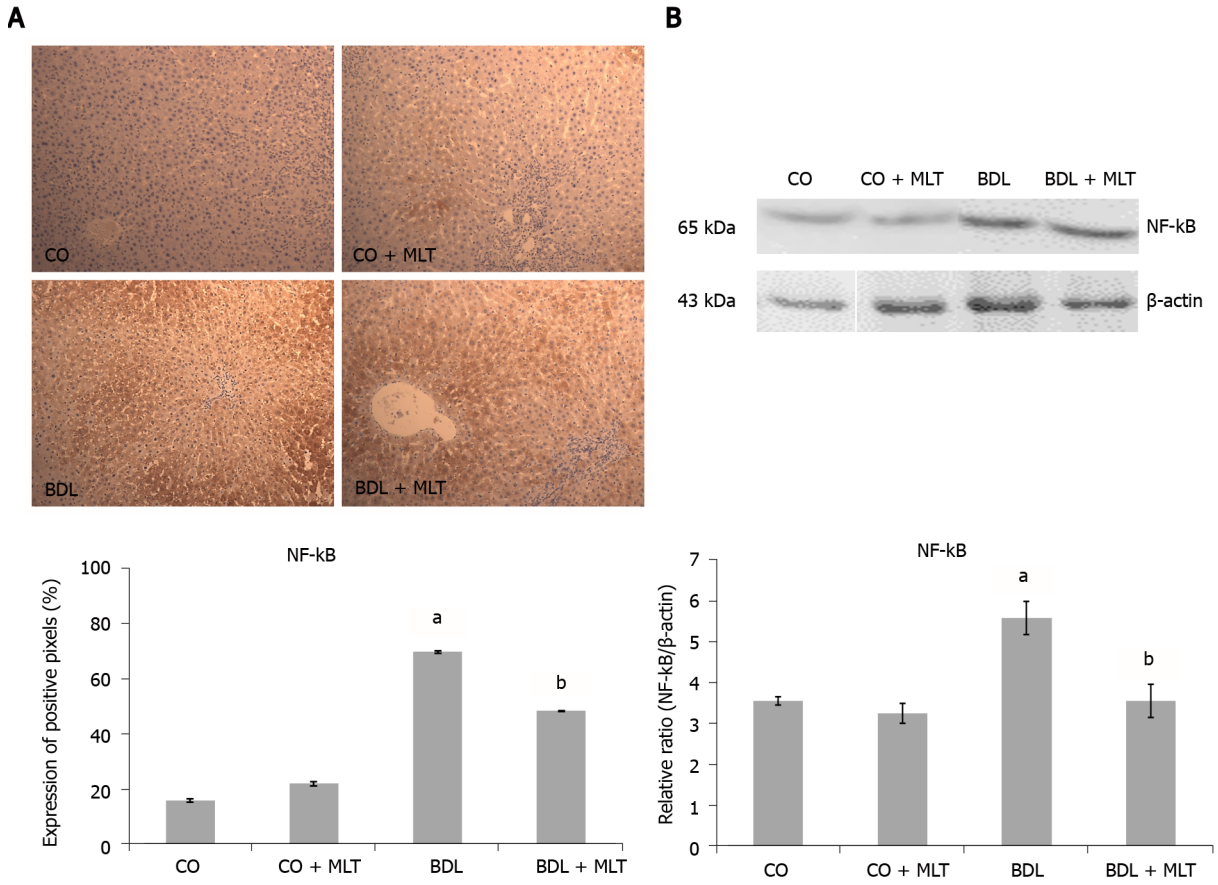


Figure 5 The effect of melatonin on the quantification of immunohistochemistry analysis and the expression of NF-kB with the Western blot technique in the livers of animals that underwent bile duct ligation surgery. The data are expressed as mean ± standard error of the mean. Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^{a}P < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^{b}P < 0.001$); A: Expression of positive pixels; B: Relative ratio (NF-kB/β-actin).

this model. The results of the present study corroborate those of Moreira *et al*[40], who used an experimental model of diethylnitrosamine-induced hepatocellular carcinoma, finding a lower damage index and frequency in the MLT-treated group, possibly due to MLT's antioxidant capacity to regulate several key genes involved in DNA repair pathways, in addition to lower oxidative damage, inflammatory processes and tissue damage, as observed in our study. The pathophysiological mechanisms of cirrhosis initiate several signaling pathways, such as the Nrf2 pathway, which has a protective effect against oxidative damage. Under stress conditions, Nrf2 is translocated to the nucleus and activates the expression of genes that encode various antioxidant enzymes, such as SOD and NQO1[17,42,50]. Nuclear expression of Nrf2 was significantly lower in the BDL group in our study. Likewise, we observed that MLT treatment significantly increased Nrf2 expression and reestablished the expression of NQO1 and SOD, which suggests that MLT restored the antioxidant system, reducing oxidative stress by modulating the Nrf2 pathway. These results were similar to those of Schemitt *et al* 2019[42], who evaluated the effects of glutamine in a severe acute liver failure model and observed increased expression of NQO1 and SOD through regulation of the Nrf2-mediated antioxidant system.

In the present study, increased pro-inflammatory cytokine IL-1β levels were observed in the BDL group, as well as increased expression of NF-kB. MLT reversed the inflammatory process, which was demonstrated by lower IL-1β levels and reduced NF-kB expression. Colares *et al*[3] found increased expression of TNF-α and inducible nitric oxide synthase in their BDL group. Inducible nitric oxide synthase is expressed in inflammatory conditions in response to pro-inflammatory cytokines and is associated with increased nitric oxide levels, which we evaluated in the present study. Our results suggest that, due to its anti-inflammatory effect, MLT modulated the NF-kB pathway during inflammation, thus reducing the expression of genes involved in the inflammatory process, such as inducible nitric oxide synthase, nitric oxide and pro-inflammatory cytokines (IL-1β and TNF-α).

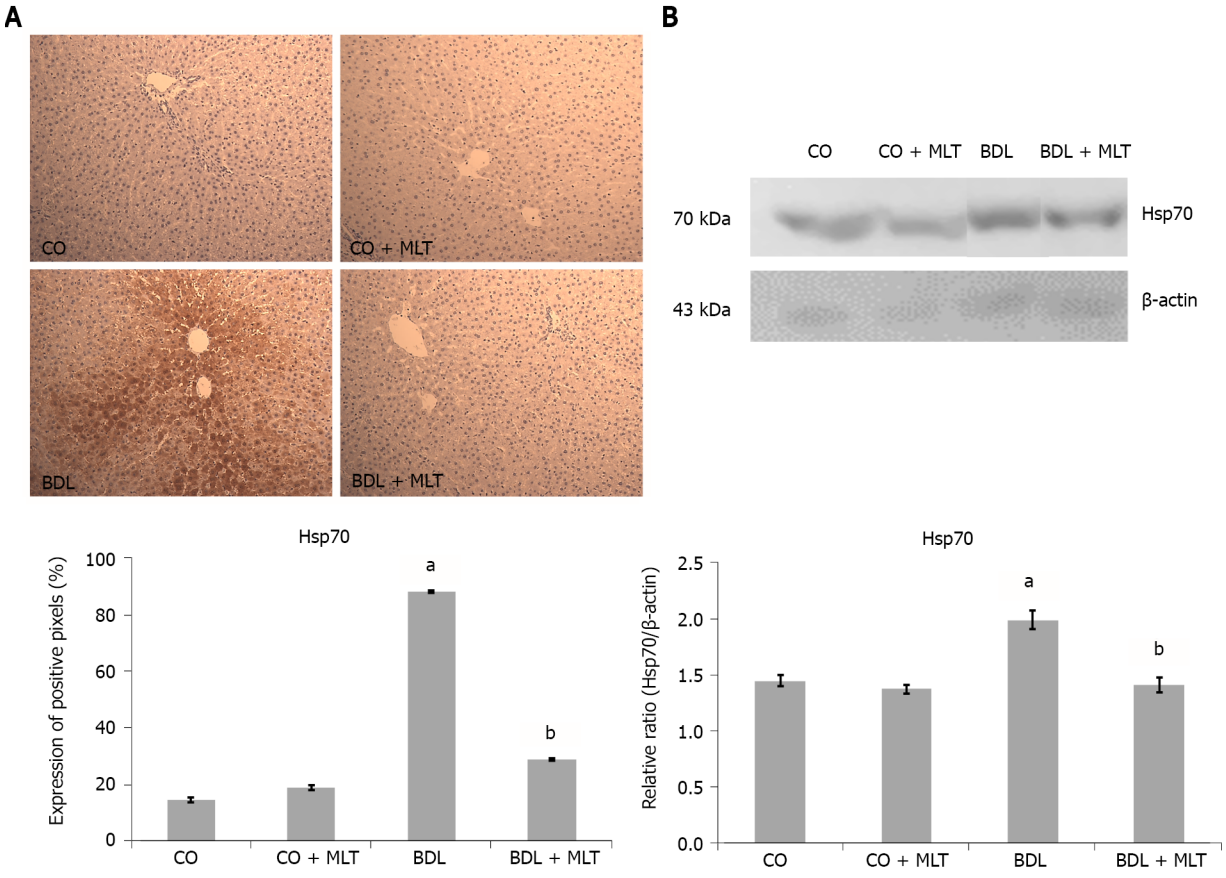


Figure 6 The effect of melatonin on immunohistochemistry and Hsp70 expression with the Western blot technique in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^aP < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$). A: The effect of melatonin on immunohistochemistry; B: Hsp70 expression with the Western blot technique.

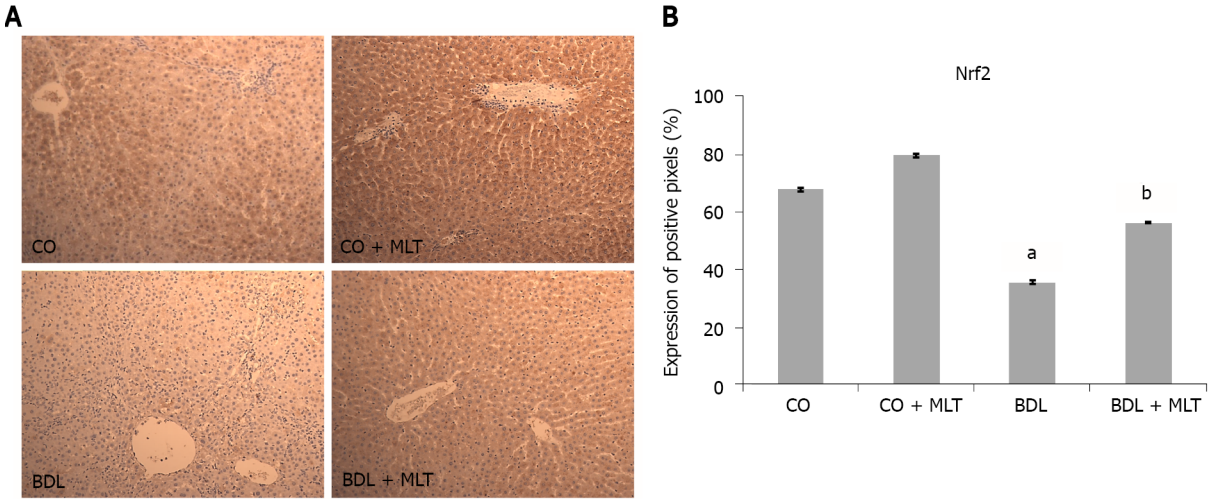


Figure 7 The effect of melatonin on Nrf2 immunohistochemistry in the liver of animals that underwent bile duct ligation surgery. The data are expressed as the mean ± standard error of the mean. A: Express the situation; B: Significant decrease in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^aP < 0.001$). Significant increase in the BDL + MLT group in relation to the BDL group ($^bP < 0.001$).

In a liver fibrosis model in rats, Czechowska *et al*[51] demonstrated that MLT inhibited the release of NF-kB and, consequently, reduced production of pro-inflammatory cytokines, probably due to its anti-inflammatory antioxidant action. In CCL4-induced liver cirrhosis model in rats, Hardeland[18] observed that MLT reduced the expression of NF-kB, as well as the expression of inducible nitric oxide synthase.

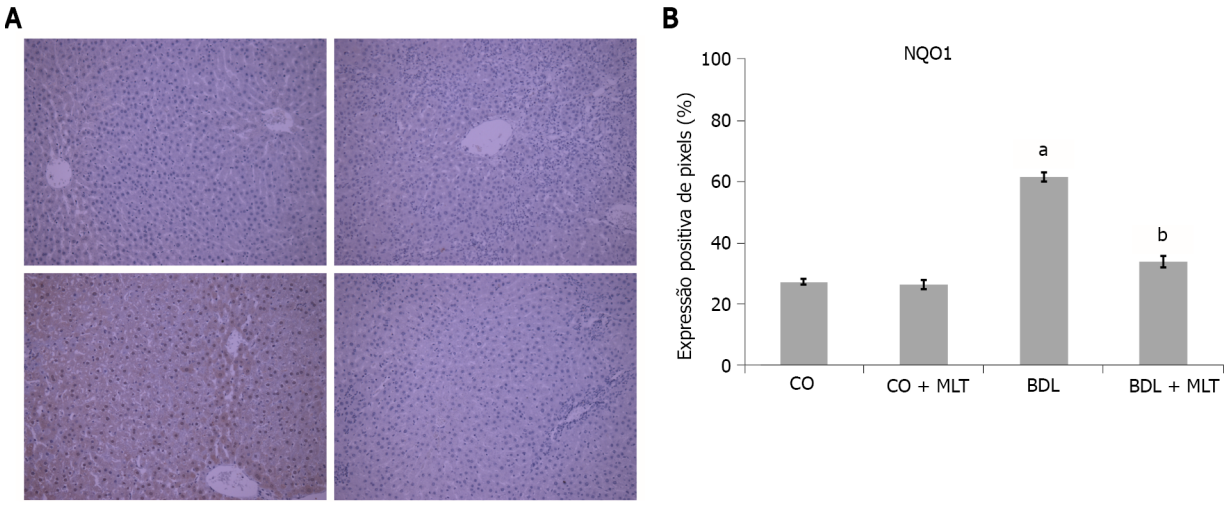


Figure 8 The effect of melatonin on quantification of NQO1 immunohistochemistry in the liver of animals that underwent bile duct ligation surgery. The data are expressed as mean ± standard error of the mean. A: Express the situation; B: Significant increase in the bile duct ligation (BDL) group in relation to the control (CO) and CO + melatonin (MLT) groups ($^{a}P < 0.001$). Significant decrease in the BDL + MLT group in relation to the BDL group ($^{b}P < 0.001$).

Heat shock proteins are extremely important for the protection of cells. In particular, Hsp70, which has cytoprotective functions, acts on protein folding, transport and degradation and can be induced in response to various stresses, including trauma, inflammatory diseases, oxidative stress and liver cirrhosis[52]. We observed an increase in the expression of Hsp70 in the animals of the BDL group, however, treatment with MLT led to a significant reduction in the expression of Hsp70. This suggests that MLT, possibly due to its important antioxidant effect, effectively regulated Hsp70 in the BDL + MLT group. In 2015, Moreira *et al*[40] reported an increase in Hsp70 expression in a model of hepatocellular carcinoma in rats treated with MLT. In 2019, Schemitt *et al*[42] evaluated a model of liver toxicity and observed that the expression of Hsp70 was reduced, possibly due to the increase in oxidative stress, thus contributing to disease worsening.

Biosynthesis of MLT by cholangiocytes is essential for maintaining biliary epithelium function and this cytoprotective mechanism, which appears to be impaired by decreased biliary MLT synthesis in biliary duct obstruction, exacerbates biliary damage and liver fibrosis. Concomitant with enhanced liver fibrosis, we observed increased biliary senescence[20].

MLT has been demonstrated to ameliorate liver damage by decreasing oxidative stress, inflammatory responses, and bile acid-induced apoptosis. The inadequate expression of MLT predisposes liver cells to immune- and oxidative stress-related damage[53].

In cholangiocytes exposed to mitochondrial oxidative stress, MLT decreased the expression of proapoptotic stimuli, which was accompanied by the inhibition of NFκB-p65, a pivotal mediator of inflammatory response, activation of antiapoptotic signaling, and increased biliary senescence and ROS, which activated HSCs by a paracrine mechanism, directly interacting with MLT on HSCs[20]. In human cholangiopathies such as PBC and PSC, an initial balance between cholangiocyte apoptosis and compensatory cholangiocyte proliferation is followed by a failure in cholangiocyte proliferative capacity, and enhanced apoptosis favors evolution toward ductopenia [20].

CONCLUSION

It can be concluded from our results that MLT treatment reduced tissue and cellular lesions in the liver, inhibited lipoperoxidation and DNA damage and reduced NO levels. In addition, MLT regulated cytoprotective capacity, regulating the Nrf2 pathway and restoring the enzymes NQO1 and SOD in the livers of treated animals.

Our results suggest that MLT has potential for clinical practice, although which patients might benefit, when treatment should begin, the dosage, and treatment duration must still be determined. Thus, larger studies assessing the efficacy and safety of MLT in the long term and in the later stages of cirrhosis are required before

its clinical use can be recommended.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis, which causes millions of deaths *per* year, is characterized by the appearance of fibrotic nodules and septa caused by chronic harmful stimuli. Oxidative damage may play a key role in the development and progression of cirrhosis. Thus, promoting the identification of new antioxidant compounds can contribute to enriching the available therapeutic arsenal.

Research motivation

Numerous studies using different experimental models have reported melatonin (MLT)'s protective effects on the liver. The antioxidant effect of MLT is related to its high solubility in lipids, which facilitates its passage through cell membranes. Thus, understanding the mechanisms involved in the protective action of MLT in cirrhosis can lead to the development of new therapeutic strategies that can lead to improved medical care.

Research objectives

The aim of the study was to evaluate the protective action of MLT in cirrhosis induced by bile duct ligation (BDL) in rats.

Research methods

Wistar rats were divided into a control group, a MLT control group, a BDL group, and a BDL group treated with MLT. Intraperitoneal administration of MLT at a dose of 20 mg/kg of body weight started on the 15th day after the beginning of the experiment and continued daily for 14 d. At the end of the experiment, the animals were euthanized. Blood was collected for liver integrity tests and the liver was collected for histological analysis, DNA damage assessment, and biochemical and Western blot analysis of proteins related to oxidative stress and the inflammatory process.

Research results

MLT promoted a significant improvement in the biochemical parameters of oxidative stress markers and the inflammatory process. DNA damage was also lower in animals treated with MLT after undergoing BDL. Tissue damage and protein expression assessed by immunohistochemistry and Western blot analysis were significantly lower in animals treated with MLT.

Research conclusions

According to the results obtained in the evaluated parameters, treatment with MLT reduced tissue and cell damage in the liver. Our results suggest that MLT may be of use of in patients with cirrhosis.

Research perspectives

Further studies are needed to assess the long-term efficacy and safety of MLT administration in cirrhotic patients before it can be recommended in clinical practice.

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ORIGINAL ARTICLE

Case Control Study

Microbiome changes in the gastric mucosa and gastric juice in different histological stages of *Helicobacter pylori*-negative gastric cancers

Qing-Hua Sun, Jing Zhang, Yan-Yan Shi, Jing Zhang, Wei-Wei Fu, Shi-Gang Ding

ORCID number: Qing-Hua Sun 0000-0001-5029-587X; Jing Zhang 0000-0003-4992-1831; Yan-Yan Shi 0000-0003-0247-371X; Jing Zhang 0000-0003-0083-3865; Wei-Wei Fu 0000-0003-3543-854X; Shi-Gang Ding 0000-0002-0831-0965.

Author contributions: Ding SG, Shi YY, and Sun QH designed the study; Sun QH analyzed the data and wrote the manuscript; Sun QH, Zhang J, Fu WW and Zhang J collected the samples and conducted the laboratory experiments; all authors approved the final version of the article.

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Qing-Hua Sun, Jing Zhang, Jing Zhang, Wei-Wei Fu, Shi-Gang Ding, Department of Gastroenterology, Peking University Third Hospital, Beijing 100191, China

Yan-Yan Shi, Research Center of Clinical Epidemiology, Peking University Third Hospital, Beijing 10019, China

Corresponding author: Shi-Gang Ding, MD, Professor, Department of Gastroenterology, Peking University Third Hospital, No. 49 Huayuan North Road, Beijing 100191, China.
dingshigang222@163.com

Abstract

BACKGROUND

The gastric microbiota in patients with gastric cancer (GC) has received increasing attention, but the profiling of the gastric microbiome through the histological stages of gastric tumorigenesis remains poorly understood, especially for patients with *Helicobacter pylori*-negative GC (HPNGC).

AIM

To characterize microbial profiles of gastric mucosa and juice for HPNGC carcinogenesis and identify distinct taxa in precancerous lesions.

METHODS

The 16S rRNA gene analysis was performed on gastric mucosa from 134 *Helicobacter pylori*-negative cases, including 56 superficial gastritis (SG), 9 atrophic gastritis (AG), 27 intestinal metaplasia (IM), 29 dysplasia (Dys), and 13 GC cases, to investigate differences in gastric microbial diversity and composition across the disease stages. In addition, paired gastric mucosa and juice samples from 18 SG, 18 IM, and 18 Dys samples were analyzed. α-Diversity was measured by Shannon and Chao1 indexes, and β-diversity was calculated using partial least squares discrimination analysis (PLS-DA). Differences in the microbial composition across disease stages in different sample types were assessed using the linear discriminant analysis effect size.

RESULTS

The diversity and composition of the bacterial microbiota in the gastric mucosa changed progressively across stages of gastric carcinogenesis. The diversity of the

authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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gastric mucosa microbiota was found to be significantly lower in the IM and Dys groups than in the SG group, and the patients with GC had the lowest bacterial community richness ($P < 0.05$). Patients with IM and those with Dys had similar gastric mucosa microbiota profiles with *Ralstonia* and *Rhodococcus* as the predominant genera. Microbial network analysis showed that there was increasing correlation strength between IM and Dys ($|correlation| \geq 0.5$, $P < 0.05$). GC and its precancerous lesions have distinguishable bacterial taxa; our results identified HPNGC-associated bacteria *Streptococcaceae* and *Lactobacillaceae* ($P < 0.05$). Additionally, across precancerous lesion stages from AG to Dys in *Helicobacter pylori*-negative patients, *Burkholderiaceae* abundance continuously increased, while *Streptococcaceae* and *Prevotellaceae* abundance presented a continuous downward trend. Furthermore, the microbial diversity was higher in gastric juice ($P < 0.001$) than in the mucosa, while PLS-DA revealed a statistically significant difference between the two groups (ANOSIM, $P = 0.001$). A significant difference in the microbial structure was identified, with *Proteobacteria* being more prevalent in the gastric mucosa and *Firmicutes* being more abundant in gastric juice.

CONCLUSION
Our results provide insights into potential taxonomic biomarkers for HPNGC and its precancerous stages and assist in predicting the prognosis of IM and Dys based on the mucosal microbiota profile.

Key Words: Gastric mucosa; Gastric juice; Microbiota; Stomach neoplasms; Histological stages; 16s RNA gene sequencing

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Core Tip: The gastric microbiome profile of *Helicobacter pylori*-negative precancerous lesions is poorly understood. This is the first study, to our knowledge, to compare the microbiota differences between paired gastric mucosa and gastric juice at different stages of gastric neoplastic progression. The findings revealed that the bacterial community of gastric juice differed from that of the gastric mucosa and that *Helicobacter pylori*-negative gastric cancer and precancerous lesions have distinct bacterial taxa. Patients with intestinal metaplasia and dysplasia had similar gastric mucosa microbiota profiles, with *Ralstonia* and *Rhodococcus* being the most predominant genera, which could aid in prognosis prediction.

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INTRODUCTION

Gastric cancer (GC) is one of the most common tumor types. Despite its declining prevalence, GC is the sixth most prevalent cancer worldwide, accounting for 8.2% of all cancer-related fatalities[1]. *Helicobacter pylori* (*H. pylori*) infection is one of the major carcinogens associated with GC[2]. In the etiology of GC, *H. pylori* is the most important pathogen in the development of GC due to atrophic gastritis (AG), which mostly results in intestinal-type GC and non-AG, which primarily results in diffuse-type GC[3]. Gastric adenocarcinoma is a complex disease associated with several different risk factors. Approximately 30% of stomach malignancies are not caused by *H. pylori* infection[4]. Heterogeneity is influenced by factors such as demographic characteristics, lifestyle, excessive salt and nitrate diet, race, and genetic variables[5-9].

Even though *H. pylori* is recognized as a class I carcinogen by the International Agency for Research on Cancer because of its association with GC, an *H. pylori*-

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negative subgroup does exist[10]. The proportion of *H. pylori*-negative GC (HPNGC) among patients with GC varies from 0.7% to 47.8% in previous reports, and a possible poorer prognosis might exist in HPNGC[11-14].

It is gradually accepted that the stomach does indeed host a robust microbiota due to breakthroughs in PCR and metagenomics methods[15]. An increasing number of studies on the link between the gastric microbiota and GC have been spurred by these technological advancements. The majority of GC cases are the intestinal type of non-cardia GC, which develops from AG to intestinal metaplasia (IM) and to GC *via* predictable progression[16]. Gastric microbiota diversity has been characterized by the severity of phenotypes, including SG, AG, IM, and GC, in many studies[15,17-19].

However, it remains unclear whether there is a correlation between the diversity of gastric microbiota and the development of gastric carcinogenesis. There is currently no consensus on the relationship between microbiota diversity and GC development stage, despite the fact that several studies have used similar methods of data collection, exclusion criteria, molecular methods for analysis, and similar measures for diversity (via Shannon's diversity index or Chao1 richness estimator). The majority of studies investigating this problem have used gene sequencing on mucosal biopsy samples collected by upper endoscopy to examine the gastric microbiota of patients with conditions ranging from normal gastric mucosa to GC[17,20-22].

Until recently, although the gastric microbiota in patients with GC has received increasing attention, only a limited number of studies have focused on patients with HPNGC and research on gastric juice microbiota between precancerous disease progression has remained relatively scarce. Prioritizing patients with HPNGC and analyzing gastric juice samples will help fill the gap in our understanding of GC. Therefore, our study focused on *H. pylori*-negative patients and performed 16S rRNA gene analysis of gastric mucosal and juice samples to determine gastric microbiome dysbiosis across stages of HPNGC and the differences in bacterial communities between gastric mucosa and juice.

MATERIALS AND METHODS

Study design and participants

Patients were recruited from the Department of Gastroenterology of Peking University Third Hospital between September 2019 and October 2020 during upper gastroenterology endoscopic examination or endoscopic submucosal dissection[A1] due to precancerous mucosal lesions. Written informed consent was obtained from all subjects in this study. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Peking University Third Hospital Medical Ethics Committee (No. IRB00006761-M2017414).

The inclusion criteria were as follows: (1) Age > 18 years; and (2) biopsy specimens and gastric juice. The exclusion criteria were: (1) Present use of antibiotics, antacids, probiotics, and prebiotics or within the last month before gastroscopy; (2) Previous gastric surgery; (3) Use of immunosuppressants; (4) Comorbidity and complications with serious heart, liver, lung, kidney, blood, endocrine, nervous system, or autoimmune diseases; (5) Bile reflux gastritis, gastroesophageal reflux disease, gastroduodenal or esophagus ulcer, or colorectal cancer; (6) A positive test for human immunodeficiency virus or hepatitis B or C virus; and (7) pregnancy or lactation. Experienced endoscopists performed all endoscopic examinations and obtained biopsy specimens and gastric juice. Demographic information, medical history, medication use, and dietary habits were collected from all subjects.

Sampling and histological evaluation

Gastric mucosal biopsy samples of 1-2 mm were obtained using standard gastroscopic forceps. A biopsy for histologic examination was performed based on the disease condition and as needed. The gastric biopsy samples for histological examination were fixed in 10% formalin and placed in separate vials, which were labeled according to their topographic site. Additional mucosal biopsy specimens were taken from the gastric antrum for microbial analysis. The biopsy specimens for microbial analysis were immediately frozen in liquid nitrogen, transferred to the laboratory, and stored at −80°C until DNA extraction. Gastric juice was drained in a sterile drainage tube at the beginning of the endoscopy. Then, the mucous material was removed by centrifugation at 4000 rpm [A2] for 10 min at 4°C, and samples were stored at -80°C until DNA extraction.

Two pathologists reviewed the gastric mucosa specimens separately according to the criteria proposed by the Chinese Association of Gastric Cancer[23] and the Updated Sydney System[24]. The diagnosis and classification of dysplasia (Dys) were determined using the revised Vienna Classification System[25]. GC was confirmed to have gastric adenocarcinoma and was divided into diffuse, intestinal, and mixed types according to the Lauren Classification. Each biopsy was diagnosed as non-atrophic superficial gastritis (SG), chronic AG, IM, or Dys based on the most severe histology. Improved Warthin-Starry (W-S) silver staining was performed on each gastric mucosa specimen. Both positive 13C-urea breath test and positive W-S staining identified the specimens as *H. pylori*-positive; otherwise, they were preliminarily identified as negative.

DNA extraction and 16s rRNA gene sequencing

Microbial genomic DNA was isolated using the E.Z.N.A® Soil DNA Kit (Omega Biotek, Norcross, GA, United States) according to the manufacturer's instructions. The V3-V4 hypervariable regions of the bacterial 16S ribosomal RNA gene were amplified using the primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min.

16S rRNA gene sequencing data processing

Raw reads of 16S rRNA gene sequences were de-multiplexed and quality-filtered using the Quantitative Insights Into Microbial Ecology (QIIME) platform[26]. Sequences were then clustered into OTUs based on 97% similarity. Using the Ribosomal Data Project Bayesian Classifier in QIIME, operational taxonomic units (OTUs) were assigned to phyla, classes, orders, families, and genera, and their relative abundances were calculated[27].

Bioinformatics analysis

Bioinformatics analyses were performed using the Majorbio cloud platform. The read counts were normalized using the total sum normalization. Based on the normalized OTU abundance profile, microbial alpha diversity was measured using the Shannon and Chao1 indices. Alpha diversity indices were compared by one-way analysis of variance (ANOVA) followed by false discovery rate (FDR) correction. The dissimilarity of the microbial communities among groups was evaluated by partial least squares discrimination analysis (PLS-DA) using R software. Sample clustering in beta diversity analysis was tested using analysis of similarity (ANOSIM) using the vegan package in R software. Relative bacterial abundances were analyzed using the Kruskal-Wallis test with FDR correction for multiple testing. The key bacterial genera responsible for discrimination between different groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) algorithm. LDA > 3.5 and $P < 0.05$ indicated significantly enriched microbial communities[28]. The microbiome analyst platform was used to explore and visualize the associations between the core microbes. Heatmaps were generated according to the relative abundance of taxa using R software (http://www.R-project.org).

Network analysis of core microbes

Spearman correlation analysis was performed to calculate the correlation coefficients (r values) between specific disease-related genera in the gastric mucosa. Two genera were connected by an edge if the correlation between them meets the P value ($P < 0.05$) and correlation threshold ($|\text{correlation threshold}| \geq 0.5$) cut-off. The Kruskal–Wallis test was conducted to compare the interaction strengths between the different gastric lesion groups. Statistical significance was set at $P < 0.05$.

Data analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, United States). Data are presented as the mean ± SEM. ANOVA was used to compare differences among groups, followed by FDR correction for multiple comparisons. Statistical significance was set at $P < 0.05$. Correlation coefficients between disease phenotype parameters and alterations in microbial taxa were analyzed using Spearman's correlation analysis.

RESULTS

Demographic characteristics of study participants

A total of 183 patients were included, including 83 patients with SG, 21 with AG, 33 with IM, 34 with Dys, and 15 with GC according to the pathological report. Furthermore, samples with < 1% *H. pylori* relative abundance were grouped as *H. pylori*-negative, while those with > 1% *H. pylori* relative abundance were grouped as *H. pylori*-positive[18]. According to this standard, 56 SG, 9 AG, 27 IM, 29 Dys, and 13 GC were confirmed as *H. pylori*-negative and enrolled in our cohort. The demographic characteristics of the subjects are shown in Supplementary Table 1.

Gastric mucosa microbiota diversity

After sequencing and quality filtering, a total of 11699206 high-quality reads were generated from all samples. The average length of the sequences was 433 bp. The data were rarefied to 7234 sequences per sample to control for variations in sequencing efforts and clustered into 2296 OTUs at 97% sequence similarity. First, to test the sequencing depth, rarefaction curves were drawn, and the sequencing data volume was sufficient (Figure 1A). The generated Venn diagram showed that 103 OTUs were shared by five groups, with 489, 91, 215, 171, and 62 OTUs unique to the SG, AG, IM, Dys, and GC groups, respectively (Figure 1B). The Shannon and Chao1 indices were used to describe the α-diversity of the gastric bacterial community. The diversity and richness of the microbial community showed a declining trend across stages of gastric carcinogenesis, from SG, AG, IM, and Dys to GC. The diversity of microbiota was significantly higher in the SG group than in the IM and Dys groups (Shannon index, P = 0.003 and 0.001, respectively), and the richness of the microbiota was significantly higher in the SG group than in the GC group (Chao1 index, P = 0.027, Figure 1C). The β-diversity analysis with PLS-DA based on the OTU level revealed a pattern in which the samples were assigned into four separate groups (ANOSIM, P = 0.005; Figure 1D). Provoked by this interesting pattern, we conducted hierarchical clustering analysis at the genus level. IM samples were divided into two condensed groups, and the same result was applied to the Dys samples (Supplementary Figure 1). The IM and Dys samples were regrouped based on a hierarchical clustering tree plot. Subgroups IM-1 and Dys-1 had a similar microbiota composition with a high relative abundance of *Ralstonia* (Supplementary Figure 2).

Mucosal bacteria changes in different histological stages of gastric carcinogenesis

The differences in the gastric mucosa microbiota between each group were investigated at different taxonomic levels. The proportion of community abundance at the phylum level was calculated and is shown in Figure 2. Firmicutes, Bacteroidota, unclassified_k__norank_d__Bacteria, and Actinobacteria were the most predominant phyla, contributing to > 90% of the microbial composition of all groups. Both IM and Dys had higher abundances of Proteobacteria than the other disease stages (P < 0.001). The clusters of IM and Dys were close to each other, suggesting a similar gastric microbiota profile. Firmicutes was more abundant in patients with GC than in those with IM and Dys (P = 0.001 for both). Bacteroidetes was less abundant in the Dys group than in the SG group (P = 0.029) (Supplementary Table 2). The clusters of IM and Dys were close to each other, suggesting a similar gastric microbiota profile.

As shown by community analysis sunburst plots at the family level, *Burkholderiaceae* (12.44%), *unclassified_k__norank_d__Bacteria* (9.34%), *Prevotellaceae* (7.98%), and *Streptococcaceae* (7.54%) were more abundant in patients with SG. *unclassified_k__norank_d__Bacteria* (26.29%), *Prevotellaceae* (9.42%), *Streptococcaceae* (9.21%), and *Lactobacillaceae* (6.42%) were the main communities in patients with AG. *Burkholderiaceae* (34.08%), *Streptococcaceae* (7.94%), *Neisseriaceae* (6.16%), and *Prevotellaceae* (5.34%) were more abundant in the IM group. *Burkholderiaceae* (34.59%), *unclassified_k__norank_d__Bacteria* (4.73%), *Prevotellaceae* (4.52%), and *Streptococcaceae* (4.30%) were more abundant in patients with Dys. In the patients with GC, *Streptococcaceae* (23.92%), *Prevotellaceae* (11.11%), *Lactobacillaceae* (8.61%), and *Burkholderiaceae* (7.41%) were the dominant families. With the precancerous lesion stages from AG to Dys, *Burkholderiaceae* abundance continuously increased, while *Streptococcaceae* and *Prevotellaceae* presented a continuous trend of decline in abundance. *Streptococcaceae* and *Lactobacillaceae* abundance was significantly higher in the GC group than in the SG group (P < 0.05) (Figure 2B).

At the genus level, the top 12 genera that showed significant differences from each other were identified (Figure 2C). Taxonomic analysis indicated that the relative abundance of *Ralstonia* and *Rhodococcus* was significantly higher in patients with IM

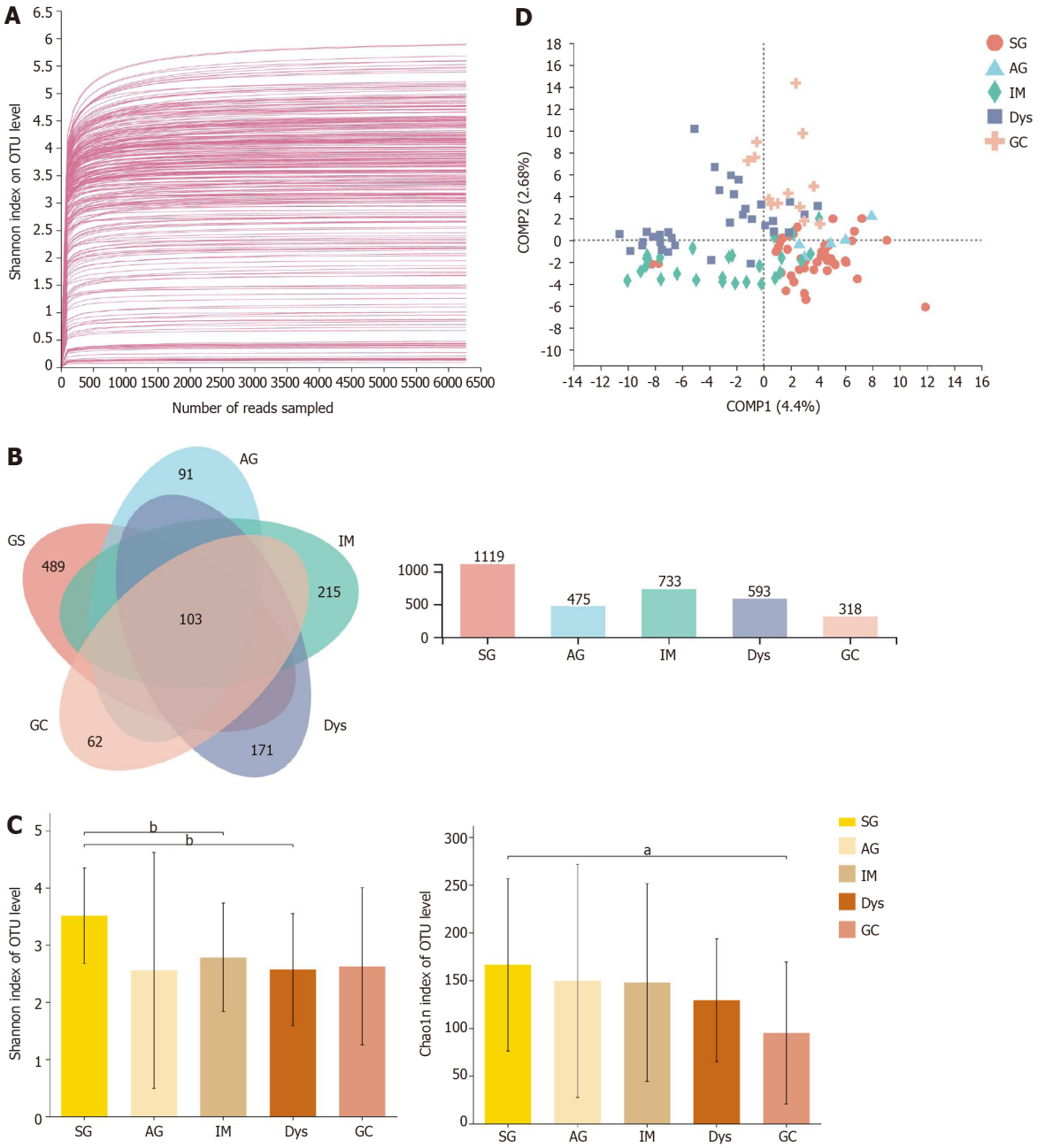


Figure 1 The microbial diversity analysis in different groups. A: Rarefaction curves of Shannon index for operational taxonomic units; B: Venn diagram; C: α-diversity indices; D: β-diversity measured by partial least squares discrimination analysis. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

and Dys than in those with SG (*Ralstonia*: $P = 0.008$ and 0.004; *Rhodococcus*: $P = 0.008$ and 0.038, respectively). *Streptococcus* and *Bifidobacterium* abundance was significantly higher in patients with GC than in those with SG ($P = 0.013$ and 0.015, respectively). *Raoultella* abundance increased in patients with Dys, and *norank_f__ mitochondria* increased in patients with AG when compared to those with SG ($P = 0.002$ and 0.008, respectively) (Table 1).

LEfSe analysis was used to identify the most relevant taxa responsible for the differences among disease stages. An LDA cutoff score of 3.5 was used to estimate the discriminatory impact of each community on the phylogenetic distribution. A total of 42 taxa were identified as key participants in the five groups (Figure 2D). Figure 2E shows the most relevant taxa responsible for the differences among disease stages at the genus level, with *Bacteroides* and *Geobacillus* identified in the SG group; *Faecalibacterium*, *Blautia*, and *norank_f__Mitochondria* in the AG group; *Rhodococcus* and *Ralstonia* in the IM group; *Enterococcus*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Raoultella* in the Dys group; and *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* in the GC group (Figure 2D).

Table 1 Relative abundance of the selected top 34 genera in different histological stages

	Relative abundance (%)						
	SG	AG	IM	Dys	GC	One-way ANOVA, *P* value	*P* value
g__Ralstonia	10.43	3.84	31.05	31.04	7.97	0.000	0.008[b]; 0.004[c]
g__Streptococcus	7.52	9.19	7.91	4.28	14.90	0.028	0.013[d]
g__Prevotella	5.81	7.83	3.82	3.40	5.70	0.111	
g__Lactobacillus	4.56	6.42	1.16	2.57	9.23	0.009	
g__Neisseria	3.80	1.05	6.03	2.81	2.36	0.538	
g__Veillonella	3.15	3.00	2.31	1.49	4.53	0.497	
g__Burkholderia-Caballeronia-Paraburkholderia	1.93	1.06	2.93	3.45	1.72	0.043	
g__Haemophilus	2.95	0.78	2.08	1.32	2.33	0.540	
g__Alloprevotella	2.05	1.54	1.51	1.06	1.39	0.373	
g__Acinetobacter	1.74	0.96	1.12	1.78	1.93	0.716	
g__Actinomyces	1.42	2.80	1.04	0.92	1.22	0.364	
g__Escherichia-Shigella	1.86	1.12	1.11	1.70	1.47	0.193	
g__Fusobacterium	1.85	0.96	1.57	0.97	1.29	0.308	
g__Pseudomonas	1.56	0.47	0.88	1.65	1.70	0.128	
g__Porphyromonas	2.14	0.53	1.38	0.78	1.32	0.259	
g__Geobacillus	1.68	0.99	0.52	1.25	1.12	0.022	
g__Bifidobacterium	0.50	1.53	0.18	0.72	2.09	0.001	0.015[d]
g__Rhodococcus	0.50	0.29	2.11	1.79	0.11	0.003	0.008[b]; 0.038[c]
g__Gemella	0.93	1.18	0.63	0.32	1.39	0.119	
g__Delftia	1.07	0.35	0.95	0.90	1.04	0.609	
g__Granulicatella	0.86	0.45	1.08	0.52	1.15	0.659	
g__Bacteroides	1.49	1.08	0.22	0.54	0.66	0.003	
g__Leptotrichia	0.66	0.73	1.02	0.49	0.65	0.689	
g__norank_f__Mitochondria	0.44	2.51	0.15	0.16	0.10	0.016	0.008[a]
g__Rothia	0.77	0.70	1.00	0.28	0.56	0.762	
g__unclassified_p__Proteobacteria	0.49	1.03	0.64	0.28	0.12	0.195	
g__Raoultella	0.03	0.02	0.81	1.60	0.00	0.000	0.002[c]
g__Sphingomonas	0.54	0.17	0.33	0.56	0.81	0.742	
g__Blautia	0.83	0.86	0.18	0.23	0.32	0.001	
g__Clostridium_sensu_stricto_1	0.24	0.43	0.30	0.81	0.54	0.292	
g__TM7x	0.57	0.48	0.63	0.28	0.26	0.269	
g__Corynebacterium	0.64	0.16	0.24	0.64	0.45	0.777	
g__norank_f__norank_o__Chloroplast	0.63	0.53	0.33	0.22	0.30	0.924	
g__Faecalibacterium	0.62	0.88	0.11	0.16	0.21	0.008	

[a]SG *vs* AG.
[b]SG *vs* IM.
[c]SG *vs* Dys.
[d]SG *vs* GC. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

Associations of specific genera and their differences between stages of gastric lesions

The relative abundance of the same 13 genera (the most relevant taxa responsible for the differences among disease stages are presented in Figure 2D) was compared among different gastric lesion groups. A network diagram was drawn based on the correlation between the genera to reflect the interactions between samples. The sizes of the nodes in the figure indicate the abundance of genera. The red color indicates a positive correlation, and green indicates a negative correlation. The thicker the line, the stronger the correlation between the genera. These results were used to visualize and identify possible associations among the important taxa. The IM and Dys groups had more complex interactions than the SG and GC groups (Figure 3). The transitivity, diameter, and average shortest path length of the different histological stages are shown in Supplementary Table 3.

The bacterial community of gastric juice was different from that of gastric mucosa

Paired-gastric juice and mucosa from 18 SG, 18 IM, and 18 Dys patients were analyzed. In the first step, we analyzed the gastric juice from patients with SG, IM, and Dys. The richness of the microbiota was significantly higher in the SG group than in the Dys group (Chao1 index, $P = 0.025$), but there were no significant differences in the diversity of microbiota between these groups (Figure 4A). Although β-diversity analysis with PLS-DA based on the OTU level revealed a pattern with three clusters, ANOSIM showed that the clusters for the three groups were not significantly different ($P = 0.230$; Figure 4B). As shown by ternary analysis at the family level, *Burkholderiaceae* was more abundant in the IM group, *Fusobacteriaceae* and *Prevotellaceae* were more abundant in the SG group, and *Veillonellaceae* and *Staphylococcaceae* were more abundant in the Dys group (Figure 4C). At the genus level, taxonomic analysis indicated that the relative abundance of *Alloprevotella* in Dys was significantly decreased, while that in IM and *Campylobacter* abundance in SG were significantly increased ($P < 0.05$, Figure 4D).

Next, the microbial α-diversity and β-diversity were measured to analyze the differences in the microbiota structure between the gastric mucosa and juice. We found that the microbial community diversity was significantly higher in gastric juice ($P < 0.001$), while there was no significant difference in microbial community richness between the two groups (Figure 5A). PLS-DA at the OTU level revealed a statistically significant separation of the groups (ANOSIM, $P = 0.001$; Figure 5B), suggesting different microbial community structures. *Proteobacteria* (59.30%), *Firmicutes* (14.37%), and *Bacteroidetes* (7.94%) were three of the most predominant phyla in the gastric mucosa, while *Firmicutes* (38.86%), *Proteobacteria* (20.01%), and *Bacteroidetes* (17.33%) were the top three most abundant phyla in gastric juice (Figure 5C).

To assess the microbiota characteristics of different stomach microhabitats, we compared pairs of gastric juice and mucosa samples for each disease stage from patients with SG, IM, and Dys. LEfSe analysis was applied to identify the most relevant taxa responsible for the differences between gastric liquid and mucosa among the disease stages (Figure 6A). We focused on bacterial taxa with different abundances at the genus and species levels. In the gastric juice of patients with Dys, enrichment in the genera *unclassified_o__Lactobacillales* and *Veillonella* was observed. In the gastric mucosa group of patients with Dys, the enriched genera were *Raoultella* and *Bacteroides*. The SG-enriched genera in the gastric mucosa were *Escherichia-Shigella* and *norank_f_Mitochondria* (Figure 6B).

DISCUSSION

In recent years, many researchers and clinicians have explored the role of the microbiome in various disease processes, which has resulted in a significant surge in the number of studies on this topic[29]. Although the severely acidic conditions of the stomach have formerly hampered research into the gastric microbiota, studies on the gastric microbiota have risen over the past decade owing to the development of modern PCR techniques and metagenomic analyses. The majority of research has compared the gastric mucosal microbiota of GC to that of SG or healthy controls without distinguishing non-*H. pylori*-infected individuals from *H. pylori*-infected ones [30-32]. Additionally, the number of studies examining the gastric microbiota utilizing gastric juice samples is still limited, and well-designed comparative studies analyzing the link between the mucosal and luminal microbiota are even rarer. To bridge these gaps, we studied the mucosal bacterial community from SG, AG, IM, Dys, and GC in

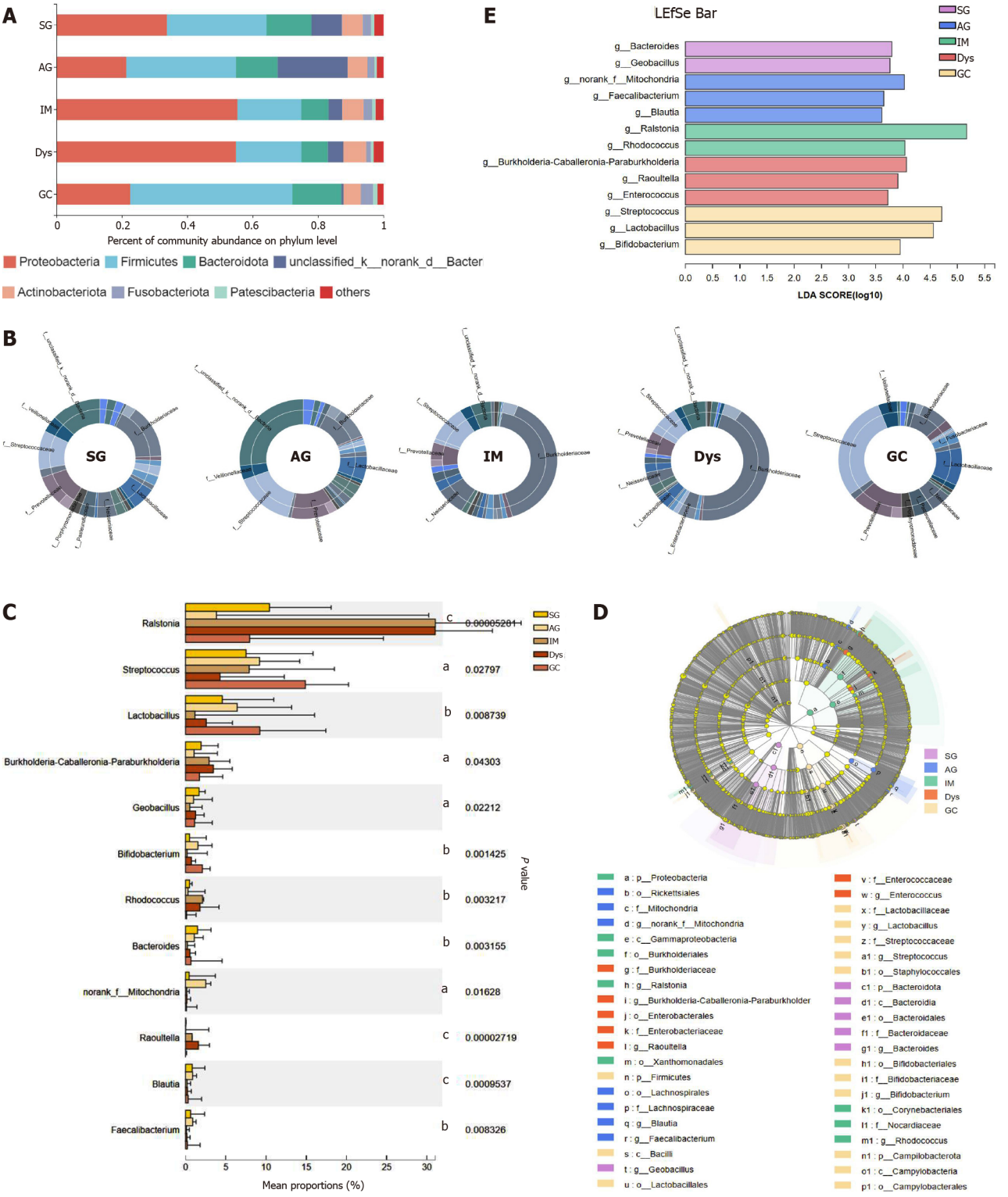


Figure 2 The mucosa microbiota composition in different groups. A: Relative abundance of phyla in five groups; B: Community analysis sunburst plot on family level; C: Changes in the gastric mucosa microbiota from superficial gastritis, through atrophic gastritis, intestinal metaplasia, dysplasia to gastric cancer; D: Linear discriminant analysis effect size (LEfSe) analysis from phylum to genus; E: Histogram of LEfSe analysis at the genus level. Significance was obtained by LEfSe (Kruskal–Wallis test) at $P < 0.05$, and linear discriminant analysis score>3.5. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. LDA: Linear discriminant analysis; LEfSe: LDA effect size; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

H. pylori-negative patients as well as the bacterial composition of gastric juice and its deviations from the mucosal microbiota.

In this study, we discovered that the α-diversity of the gastric mucosa microbiota was significantly lower in the IM and Dys groups than in the SG group using the Shannon index, and that the bacterial community richness was lowest in the patients with GC, which is supported by earlier research results[19,30,32].

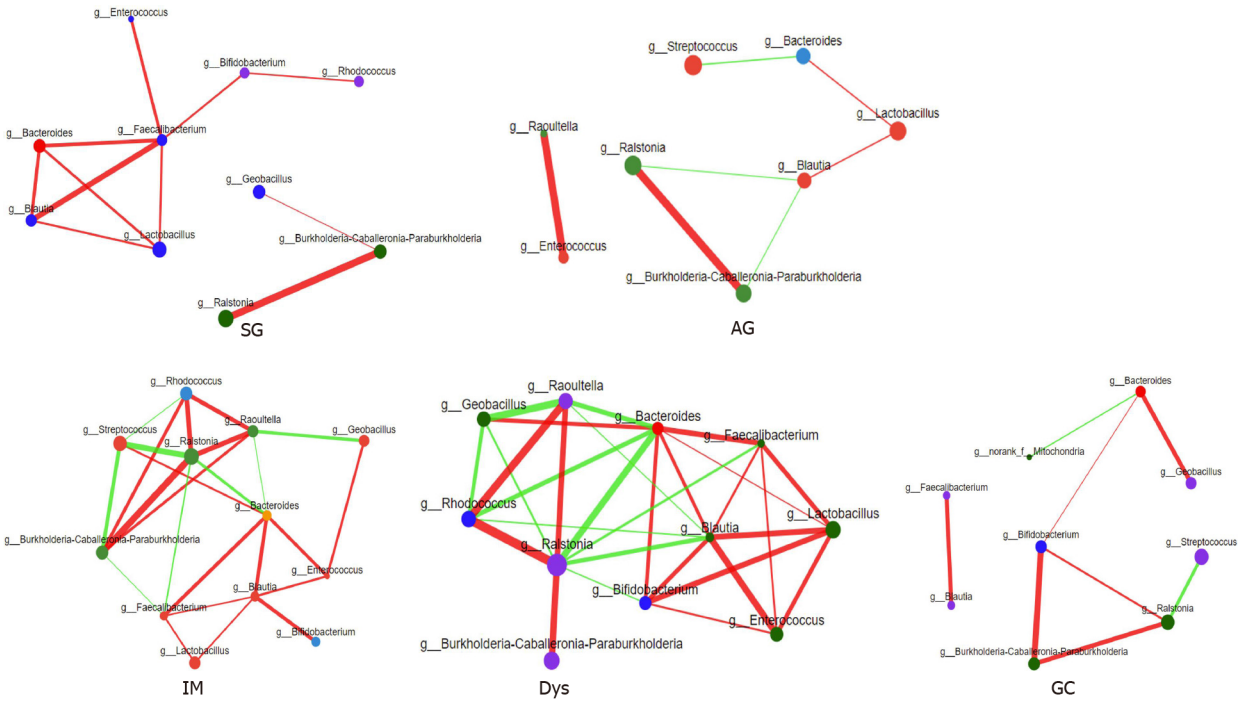


Figure 3 Correlation analysis between core microbes. SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

Our study's findings on microbial β-diversity revealed that the SG, IM, Dys, and GC groups could be distinguished from each other, which is in agreement with the results of previous studies showing that there is a shift in the composition of the microbial community along different histological phases of stomach neoplastic progression[18,33]. Notably, the AG group was intermingled with the SG group in the β-diversity plot (Figure 1D), indicating that the microbial profile of AG may be comparable to that of SG. Another possibility is that a comprehensive assessment of the microbiome in AG is not feasible owing to the sample size constraint. Another interesting finding was that patients with IM were dispersed throughout the SG and Dys as a bridge in the PLS-DA (Figure 1D). Similarly, the distribution region of β-diversity in the Dys group exhibited distinct characteristics: one portion heavily overlapped with the GC group, while the other part was near the IM group. Based on the above findings, we further plotted hierarchical clustering trees and found that there were two distinct clusters in the IM and Dys groups. We then investigated the bacterial composition of patients with IM and Dys and discovered that both groups could be divided into two sub-groups that corresponded to their β-diversity distribution (Supplementary Figure 1).

As precancerous lesions, IM and Dys have been considered intermediate stages between cancer and gastritis, and consecutive alterations in the microbiota may play a role in the progression of mucosal precancerous lesions. In clinical practice, it is challenging for digestive endoscopists to choose the appropriate interval and frequency of endoscopic follow-up for patients with IM or Dys. According to our findings, one possible solution to this problem is to use the microbiota profile of the gastric mucosa to determine whether a patient has a more cancer- or gastritis-like microbiota, allowing cancer-like patients to undergo more rigorous and frequent endoscopic monitoring or magnifying endoscopy because they may be at a higher risk of cancer. It might be more reasonable to assess gastric lesions by pathological reports combined with gastric microbiota profiles. Further research with a larger sample size is needed to validate this theory.

Streptococcus was found to be significantly more abundant in patients with GC than in those with SG, which is supported by the findings of multiple studies that used mucosal samples[33,34]. Interestingly, several investigations have indicated a large increase in *Streptococcus* abundance in malignant tissues compared to normal tissues from the same patients with GC, and similar findings have also been drawn using fecal samples, which made us more certain that *Streptococcus* should play an essential role throughout the process of gastric cancerization[35-37]. One plausible explanation is that *Streptococcus* antigens might induce cancer, since *Streptococcus bovis* has previously been shown to have such an effect in two studies using animal models[38,39].

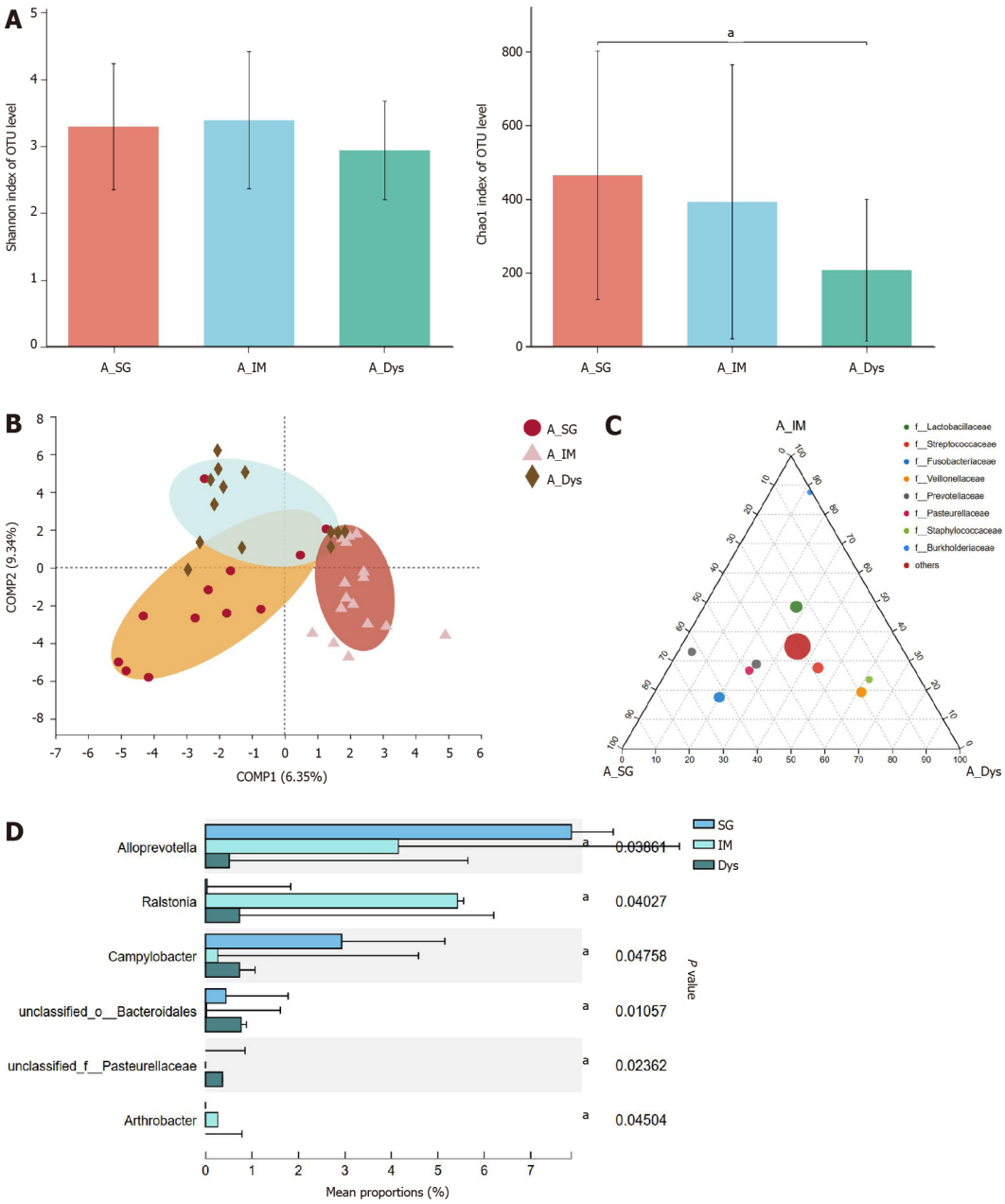


Figure 4 The microbial diversity analysis and microbiota composition of gastric juice in different groups. A: α-diversity indices; B: β-diversity measured by partial least squares discrimination analysis; C: Ternary analysis at the family level; D: Taxonomic analysis at the genus level. $^{a}P < 0.05$. SG: Superficial gastritis; IM: Intestinal metaplasia; Dys: Dysplasia.

In our study, the relative abundance of *Alloprevotella* was shown to decline significantly lower in patients with Dys than in those with SG at the genus level ($P < 0.05$, Figure 4D). This result is in-line with that of a recent study, which indicated that *Alloprevotella* levels are significantly lower in the IM/DYS group than in the normal/SG group[40]. *Alloprevotella* is known to have anti-inflammatory properties, which may explain this outcome to some degree[41,42]. In addition, *Ralstonia* abundance was found in our study to be significantly increased in the IM and Dys groups compared to in the SG group, which is consistent with the results of an earlier study[43]. *Ralstonia* has been shown to play a role in the initiation of inflammation, which explains why there was an increase in relative abundance[44]. In addition, *Ralstonia* and *Helicobacter* were verified as the top two genera of discriminant abundance in the stomachs of patients with GC, which warrants deeper analysis of the association between these two genera and GC[45].

In most clinical trials, intragastric bacterial overgrowth is examined using gastric juice culture and rarely *via* gastric mucosal tissue. Gastric juice samples are easier to collect, generally non-invasive compared to mucosal tissues, and exhibit integrated properties. They have been used to characterize the gastric microbiota in some studies

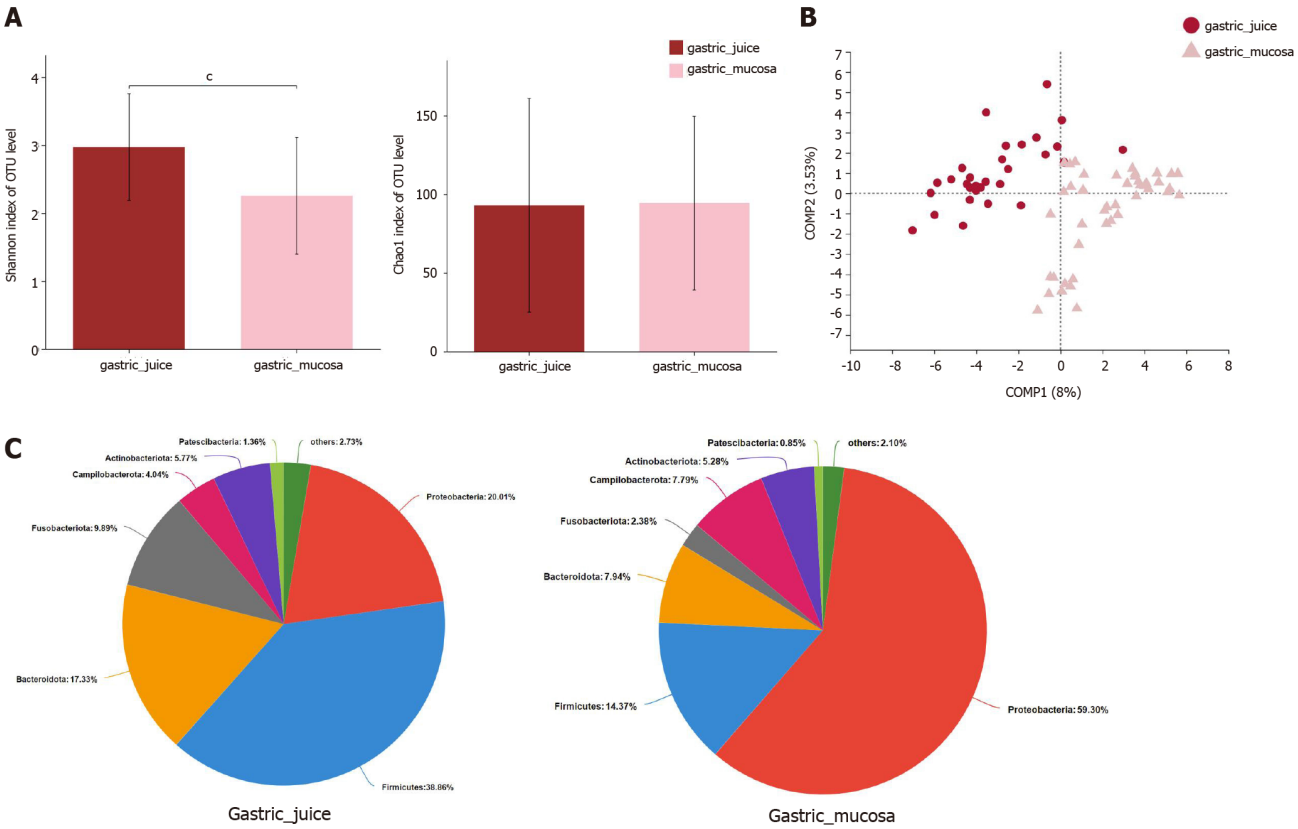


Figure 5 The microbial diversity and microbiota composition in different groups. A: α-diversity indices; B: β-diversity measured by partial least squares discrimination analysis; C: Relative abundance of phyla in two groups. °P < 0.001; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

[46,47]. In general, gastric juice samples include a combination of mucosal microbes and luminal communities[48], which have not been previously assessed in patients with GC. It has been demonstrated that oral or fecal commensal flora are usually found in the gastric juice of patients with GC[22], which indicates that there might be differences between the microbiota in gastric juice and mucosa. With respect to the influence of sample type, it was demonstrated in our study that the alterations of microbiota in gastric mucosa and gastric juice showed a discrepancy despite several earlier studies showing that microbial communities of different anatomical gastric positions are similar[18,20,36], which illustrated that gastric sample type may be a factor influencing research results, and that juice and mucosal samples should be treated separately. Future studies are still needed to confirm the differences between the mucosal microbiota and the gastral cavity microbiota.

It is a pity that only gastric juice samples in SG, IM, and Dys groups were available when samples were collected with a lack of data from the GC group. Another shortcoming is that the sample size of the AG groups was relatively small, which might not reflect the bacterial composition to the fullest. It has also been shown that tea drinking as well as fresh vegetable and fruit intake might play a role in slowing carcinogenic progression[49], which might have some influence on gastric microbiota. A detailed dietary questionnaire would ensure more rigorous and well-founded results. In-depth research on the pathogenic mechanisms of non-*H. pylori* bacteria in gastric carcinogenesis will be strongly desired in the future.

CONCLUSION

Our study showed a shift in the gastric microbial community structure along the SG-AG-IM-Dys-GC stages in the *H. pylori*-negative stages. The diversity and composition of the gastric mucosal microbiota altered gradually across the stages of gastric neoplastic progression. Patients with IM and Dys had similar gastric mucosa microbiota profiles, and their potential to be indicators of IM and Dys prognosis needs to be verified in further studies. Our findings also revealed that the bacterial

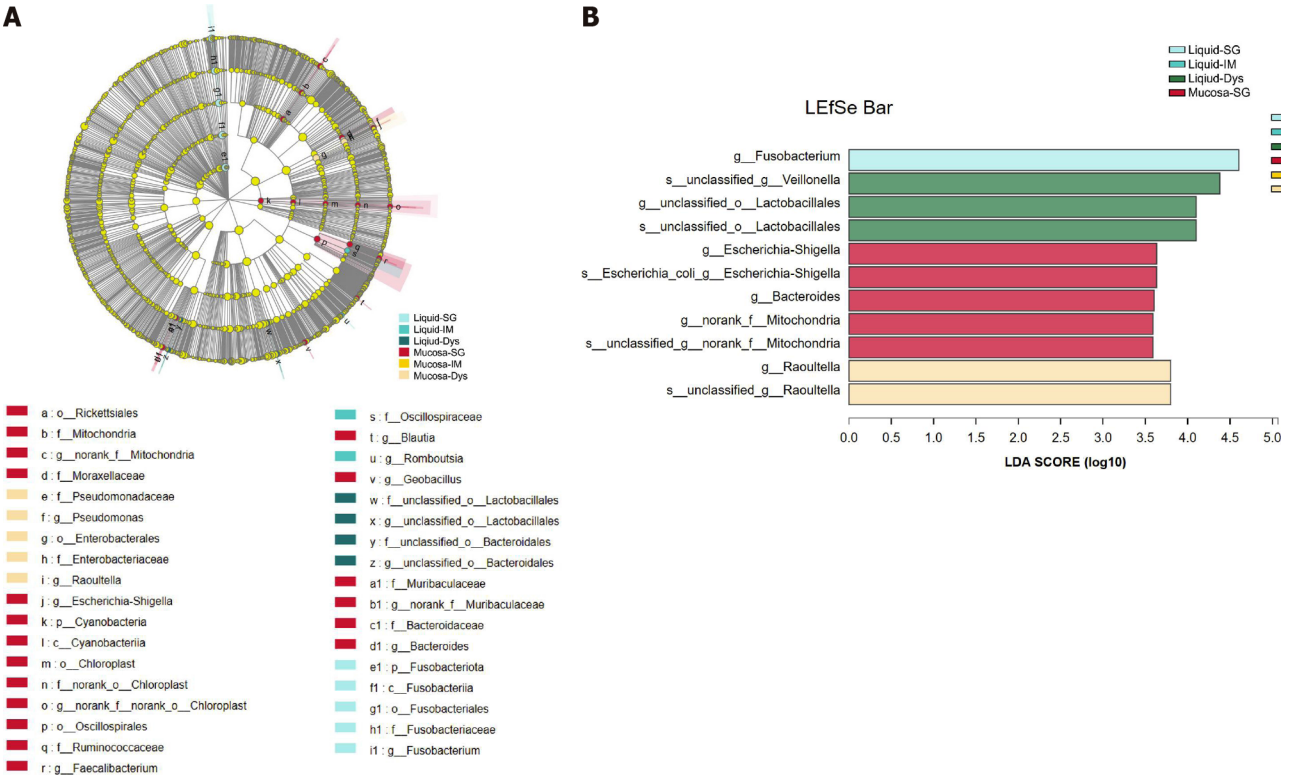


Figure 6 Linear discriminant analysis scores for differentially abundant taxonomic features among six groups. Significance was obtained by linear discriminant analysis effect size (LEfSe) (Kruskal-Wallis test) at $P < 0.05$, and linear discriminant analysis score > 3.5. A: LEfSe analysis from phylum to genus; B: Histogram of LEfSe analysis at the genus level. LDA: Linear discriminant analysis; LEfSe: LDA effect size; SG: Superficial gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; Dys: Dysplasia; GC: Gastric cancer.

community of gastric juice differed from that of the gastric mucosa, and that HPNGC and its precancerous lesions have distinct bacterial taxa. *Streptococcaceae* and *Lactobacillaceae* were enriched in HPNGC. In addition, from AG to Dys, *Burkholderiaceae* abundance increased continuously, while *Streptococcaceae* and *Prevotellaceae* presented a continuous downward trend in abundance, which suggested that *Burkholderiaceae*, *Streptococcaceae*, and *Prevotellaceae* might play different roles in the carcinogenesis of HPNGC.

ARTICLE HIGHLIGHTS

Research background

The gastric microbiome through the histological stages of gastric tumorigenesis remains poorly understood, especially for the *Helicobacter pylori*-negative gastric cancer (HPNGC).

Research motivation

To get a better knowledge of gastric microbiota and to identify microbial indicators at different histological stages of gastric tumorigenesis.

Research objectives

To identify distinct taxa in precancerous lesions and describe microbial profiles of gastric mucosa and juice for HPNGC carcinogenesis.

Research methods

We designed a clinical cohort study and utilized the 16S rRNA gene sequencing analysis.

Research results

Our study showed a change in the gastric microbial community structure along the

precancerous lesions in the *Helicobacter pylori*-negative stages. Patients with intestinal metaplasia and dysplasia had similar gastric mucosa microbiota profiles, and their potential to be indicators for prognosis. Our findings revealed that the bacterial community of gastric juice differed from that of the gastric mucosa, and that HPNGC and its precancerous lesions have distinct bacterial taxa.

Research conclusions

Using the gastric microbiota profile, we were able to identify possible taxonomic biomarkers for HPNGC and its precancerous phases, as well as help predict prognoses for intestinal metaplasia and dysplasia.

Research perspectives

Our research revealed the core pathogenic bacteria in *Helicobacter pylori*-negative precancerous lesions, allowing for further investigation of the pathogenic process.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Postoperative mortality and morbidity after D2 lymphadenectomy for gastric cancer: A retrospective cohort study

Giuseppe Brisinda, Maria Michela Chiarello, Anna Crocco, Neill James Adams, Pietro Fransvea, Serafino Vanella

ORCID number: Giuseppe Brisinda 0000-0001-8820-9471; Maria Michela Chiarello 0000-0003-3455-0062; Anna Crocco 0000-0003-2771-7821; Neill James Adams 0000-0002-2813-8648; Pietro Fransvea 0000-0003-4969-3373; Serafino Vanella 0000-0002-6599-8225.

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Giuseppe Brisinda, Abdominal Surgery, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome 00168, Italy

Giuseppe Brisinda, Department of Medical and Surgical Sciences, Università Cattolica del Sacro Cuore, Rome 00168, Italy

Maria Michela Chiarello, General Surgery Operative Unit, Azienda Sanitaria Provinciale di Crotone, Ospedale San Giovanni di Dio, Crotone 88900, Italy

Anna Crocco, Endocrine Surgery Operative Unit, Istituto Nazionale Tumori, IRCCS Fondazione Pascale, Napoli 80100, Italy

Neill James Adams, Health Sciences, Clinical Microbiology Unit, Magna Grecia University, Catanzaro 88100, Italy

Pietro Fransvea, Department of Emergency Surgery and Trauma Center, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome 00168, Italy

Serafino Vanella, Department of General and Oncological Surgery, Azienda Ospedaliera San Giuseppe Moscati, Avellino 83100, Italy

Corresponding author: Giuseppe Brisinda, MD, Fondazione Policlinico Universitario A Gemelli IRCCS, Largo Agostino Gemelli 8, Rome 00168, Italy. gbrisin@tin.it

Abstract

BACKGROUND

Surgery for gastric cancer is a complex procedure and lymphadenectomy is often mandatory. Postoperative mortality and morbidity after curative gastric cancer surgery is not insignificant.

AIM

To evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer.

METHODS

A retrospective analysis of clinical data and pathological characteristics (age, sex, primary site of the tumor, Lauren histotype, number of positive lymph nodes resected, number of negative lymph nodes resected, and depth of invasion as

data used are present in the text. No additional data are available.

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defined by the standard nomenclature) was conducted in patients with gastric cancer. For each patient we calculated the Kattan's score. We arbitrarily divided the study population of patients into two groups based on the nomogram score (< 100 points or ≥ 100 points). Prespecified subgroups in these analyses were defined according to age (≤ 65 years or > 65 years), and number of lymph nodes retrieved (≤ 35 lymph nodes or > 35 lymph nodes). Uni- and multivariate analysis of clinical and pathological findings were performed to identify the factors affecting postoperative mortality and morbidity.

RESULTS
One-hundred and eighty-six patients underwent a curative R0 resection with D2 lymphadenectomy. Perioperative mortality rate was 3.8% (7 patients); a higher mortality rate was observed in patients aged > 65 years (P = 0.002) and in N+ patients (P = 0.04). Following univariate analysis, mortality was related to a Kattan's score ≥ 100 points (P = 0.04) and the presence of advanced gastric cancer (P = 0.03). Morbidity rate was 21.0% (40 patients). Surgical complications were observed in 17 patients (9.1%). A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested (P = 0.0005).

CONCLUSION
Mortality and morbidity rate are higher in N+ and advanced gastric cancer patients. The removal of more than 35 lymph nodes does not lead to an increase in mortality.

Key Words: Gastric cancer; Total gastrectomy; Subtotal gastrectomy; Lymphadenectomy; Kattan's nomogram; Mortality; Postoperative complications; Postoperative pancreatic fistula; Hemoperitoneum; Anastomotic leakage

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Core Tip: Surgery for gastric cancer is a complex procedure. The aim of our study is to evaluate the factors determining mortality and morbidity in 186 patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer. Perioperative mortality rate was 3.8%; a higher mortality rate was observed in patients aged > 65 years and in N+ patients. Mortality was related to a Kattan's score ≥ 100 points and the presence of advanced gastric cancer. Morbidity rate was 21.0%. Surgical complications were observed in 17 patients. A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested.

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INTRODUCTION

Although the incidence of gastric cancer is steadily declining, the disease remains the second leading cause of cancer death[1,2]. Currently, surgery is the only potentially curative treatment for gastric cancer[3,4]. The depth of primary tumor invasion, lymph node involvement, and distant metastasis are the major predictors of prognosis for patients with gastric cancer[5].

Node metastases occur during the early stages of the disease, and lymphadenectomy is recommended as the main intervention of a radical surgical treatment[4,6,7]. According to the TNM staging system proposed by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC)[8], the N stage is classified into 5 Levels based on the number of metastatic lymph nodes. However, the extent of lymphadenectomy, which aims to achieve the highest optimal outcome, has been a controversial topic for a long time with no worldwide consensus

as of yet[9]. A minimum of 16 lymph nodes has been recommended as an adequate number in radical gastrectomy for gastric cancer to ensure reliable N staging. Studies have shown that the number of dissected metastatic lymph nodes influences prognosis [10].

Gastric cancer surgery is a complex procedure; in this context, lymphadenectomy is mandatory[11-14]. Mortality and morbidity after curative gastric cancer surgery are not negligible[15-17]. There are many clinical and pathological factors that induce an increase in mortality and morbidity[18]. The extent of the lymphadenectomy is one of these factors. The development of postoperative complications, and the associated mortality, is also influenced by the stage of the disease, the number of lymph node metastases, the removal of contiguous organs and the age of the patient.

In this paper, we evaluated patients with histologically confirmed gastric adenocarcinoma, who underwent curative gastrectomy and D2 lymphadenectomy according to the Japanese Gastric Cancer Association (JGCA) guidelines[19,20]. The primary endpoint of the study is to evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer. For each patient we calculated the Kattan's score. In agreement with the original report by Kattan *et al*[21] the following prognostic variables were assembled for use in validating the nomogram: age, sex, primary site [distal one-third, middle one-third, proximal one-third, and gastroesophageal junction (GEJ)], Lauren histotype (diffuse, intestinal, mixed), number of positive lymph nodes resected, number of negative lymph nodes resected and depth of invasion as defined by the standard nomenclature. We arbitrarily divided the study population of patients into two groups based on the nomogram score (< 100 points or ≥ 100 points). Prespecified subgroups in these analyses were defined according to age (≤ 65 years or > 65 years) and number of lymph nodes retrieved (≤ 35 lymph nodes or > 35 lymph nodes). The cut off was used in this study since age > 65 years is considered a significant risk factor for postoperative complications in gastric surgery, and was also in accordance with a definition of age limits for elderly patients. Clinical factors and pathological findings were evaluated to identify the factors that induce increased postoperative mortality and morbidity in patients undergoing R0 surgery. Treatment factors were also analyzed for their impact on mortality and morbidity.

MATERIALS AND METHODS

This is a retrospective study. An analysis of clinical data and pathological characteristics was conducted on patients with gastric cancer observed and treated at the General Surgery Operative Unit, Fondazione Policlinico Universitario "A Gemelli" IRCCS, from January 2010 to December 2015, and at the General Surgery Operative Unit, San Giovanni di Dio Hospital, Azienda Sanitaria Provinciale Crotone, from January 2016 to June 2020.

All patients provided written consent before the surgical procedures. Preliminary approval to use patient data was obtained from the Institutional Review Board. This study was conducted according to the STROBE guidelines[22].

Inclusion criteria

Patients with histologically documented gastric cancer were included in the study. All patients underwent a complete clinical evaluation, including laboratory tests, with complete blood cell count and serum chemistry. In all patients, a preoperative staging of the neoplasm was performed. This included upper digestive endoscopy with biopsy, chest X-ray, liver ultrasound and abdomino-pelvic CT-scan. Tumors were staged according to the latest version of the pathologic classification (pTNM) of the International UICC. The histological classification followed the Lauren criteria[23].

Exclusion criteria

Gastric stump and linitis plastica type tumors were excluded from the analysis. Patients with squamous cell cancer or stromal tumors and patients in preoperative neoadjuvant treatment protocols were also excluded from the analysis. Patients with positive surgical resection margins, patients with peritoneal carcinomatosis and/or patients with metastatic disease, and patients with > 1 missing data were not included in the study.

Surgical rules

Gastrectomy is defined by the removal of the greater and lesser omentum and

perigastric lymph nodes (N1 level, station numbers 1-6). Lymphadenectomy is classified as D2 according to the guidelines of the Japanese Gastric Cancer Association [24]. D2 lymphadenectomy involves the en-bloc removal of lymph node stations 7, 8a and 8p, 9, and 11p and 11d. The left gastric artery was suture ligated at its origin. Lymphadenectomy of the splenic hilum (station 10) was always performed. Hepatoduodenal ligament nodes (station numbers 12a, 12b, 12p) were also dissected. Cholecystectomy was performed in all patients. The resection was extended to the distal esophagus when required by tumor spread and location, which was the case in nearly all of the tumors located at the GEJ. Each lymph node station was removed and classified either during the operation or from the surgical specimen; single lymph nodes were retrieved in the fresh specimen and then submitted to histopathological examination.

For reconstruction, the Roux-en-Y technique was performed in all cases. After total gastrectomy, esophagojejunostomy, using an EEA stapler (diameter 25 mm) was used routinely. In case of a subtotal gastrectomy, Roux-en-Y gastrojejunostomy was performed using an EEA stapler (diameter 25 mm) or a linear stapler (60 mm), at surgeon's discretion. A trans-anastomotic tube was placed in all patients.

Pathological data

The surgical specimens and lymph nodes were assessed by pathologists and were classified according to the 8th Edition of the UICC/AJCC TNM staging system[8]. The T category was used to assess the depth of invasion. For nodal staging, involvement of lymph nodes was defined as follows: N0, no regional lymph nodes metastasis; N1, metastasis in 1 to 2 regional nodes; N2, metastasis in 3 to 6 regional lymph nodes; N3a, metastasis in 7 to 15 regional lymph nodes; N3b, metastasis in > 16 regional lymph nodes. Based on definitive pathological findings, the potentially curative procedures were classified as radical (R0 - microscopic tumor free) or as R1 (microscopic residual disease) according to the absence or presence of residual tumor. Palliative resection was classified based on R2 (macroscopic disease left behind)[24]. Frozen sections were not routinely used in the evaluation of margins, but only in the suspicion of a possible tumor infiltration.

Postoperative course

Antibiotic prophylaxis was used in all patients. Low molecular weight heparin treatment was used in all patients for 30 d. All patients were mobilized on the first postoperative day. The bladder catheter was removed on the first postoperative day except in clinical emergencies. The ERAS protocol was not used in any patient. The anastomosis was routinely checked prior to the patient resuming oral intake with a radiological examination using water-soluble contrast on postoperative day 4-7. The trans-anastomotic tube was removed after performing the radiological control if no sign of anastomotic leak was observed. The patients were monitored for 30 d postoperatively for complications and mortality. Complications were considered when occurring within 30 d from surgery, and with a Clavien-Dindo severity grade 2 or more[25]. Anastomotic leakage was defined as a full thickness gastrointestinal defect involving esophagus, anastomosis, staple line, gastric or jejunal stump irrespective of presentation or method of identification; an abscess close to the anastomoses is also considered as anastomotic leakage.

The patients follow up was standardized as follows: clinical examination, full blood tests and dosage of tumor markers, chest X-ray and abdominal ultrasound every 3 mo for the first 2 years and every 6 mo for the following 3 years. Digestive endoscopy and total-body CT scan were performed annually, unless otherwise required. The evaluation of the nutritional status was managed by specialized nutritionists. No patients were lost to follow-up procedure. All patients with positive lymph nodes were treated with systemic adjuvant chemotherapy.

Statistical analysis

The clinicopathological characteristics included the patient age, sex, resection type, associated splenectomy, tumor site, histological type, T category, N stage, number of lymph nodes examined, number of metastatic lymph nodes, stage of disease, depth of the primitive tumor and Kattan score. Data are expressed as a mean ± SD. Data were analyzed with standard statistical methods using GraphPad Prism Software (GraphPad, CA, United States). Comparison of means ± SD was performed with the two tailed *t*-test. A univariate analysis with all the demographic data and pathologic factors using the Fisher's exact test for categorical data and the ANOVA test for continuous data was performed. Subsequently, a multivariate logistic regression was

performed. Regardless of the used test, a P value < 0.05 was considered statistically significant.

RESULTS

During the study period, a total of 304 patients with gastric cancer were treated at the General Surgery Operative Unit, Fondazione Policlinico Universitario "A Gemelli" IRCCS of Rome, and at the General Surgery Operative Unit, San Giovanni di Dio Hospital, Azienda Sanitaria Provinciale of Crotone. Among them, 186 patients (61.2%) underwent a macroscopic potentially curative D2 lymphadenectomy (R0 resection) and were retrospectively analyzed for this observational study. The other 118 patients were excluded from the evaluation for the presence of distant metastases (50 cases, 16.4%), peritoneal carcinosis (44 cases, 14.4%) diagnosed preoperatively either by laparoscopy (31 cases) or by exploratory laparotomy, or due to R2 surgery (24 cases, 7.9%).

Demographics and intraoperative data

The main demographic data and clinical characteristics of all patients are reported in Table 1. One hundred and eight patients were male (58.1%) and 78 females (41.9%). The mean age was 64.9 ± 12.4 years (range: 24-90 years). One hundred and six patients were older than 65 years (57.0%) and 80 less than or equal to 65 years (43.0%). The mean tumor size was 4.4 ± 2.3 cm (range 0.5-14 cm). With regards to tumor localization a higher percentage of tumors were in the middle or lower third (31.2% and 43.5%, respectively) of the stomach. As far as UICC/AJCC stage groupings, 95 patients (51.0%) were in early stage of the disease (stage IA, IB, IIA) and 91 patients (49.0%) had advanced disease (stage IIB, IIIA, IIIB, IIIC). Only 40 patients (T1a 36 cases - 19.3%, T1b 4 cases - 2.1%) had early gastric cancer (Table 1). Kattan score was 117.8 ± 45.7 points (range 11-215).

Total gastrectomy was performed in 88 patients (47.3%) and subtotal gastrectomy in 98 (52.7%). Mean age of patients undergoing total gastrectomy was 63 ± 12.1 years and 66.6 ± 12.5 years in those undergoing subtotal gastrectomy (P = 0.04). In the total gastrectomy patient's subgroup, the mean Kattan score was 111.3 ± 44.1 points, statistically lower (P = 0.03) than that observed after subtotal gastrectomy (125.1 ± 46.7 points). The mean tumor size was 4.6 ± 2.6 cm (range 1-14) and 4.1 ± 2.0 cm (range 0.5-11) in patients undergoing total gastrectomy and subtotal gastrectomy, respectively (P = 0.1).

To obtain an R0 resection, adjacent organs were removed in 5 patients (2.7%): in two cases an atypical liver resection was performed, and in 3 a transverse colon resection was performed. A mean number of 38.3 ± 10.9 lymph nodes (range 17-98) were dissected. The average number of positive lymph nodes was 4.2 ± 6.3 (range 0-39). 74 patients were N0. The mean number of lymph nodes removed was 40 ± 10.4 (range 25-93) and 36.7 ± 11.1 (range 17-98) in total gastrectomy and subtotal gastrectomy, respectively (P = 0.03). The number of positive lymph nodes was 4.9 ± 6.9 (range 0-39) in patients undergoing total gastrectomy and 3.5 ± 5.7 (range 0-31) in patients undergoing subtotal gastrectomy (P = 0.1). Lymphadenectomy of the splenic hilum involved splenectomy in 105 cases (56.4%) and was performed with the spleen-preserving technique in the remaining 81 cases (43.6%). 103 patients (55.3%) had > 35 lymph nodes retrieved. Mean duration of surgical procedures was 260 ± 76.1 minutes. Mean length of postoperative hospital stay was 12.7 ± 8.2 d.

Mortality

Perioperative mortality rate was 3.8% (7 patients). Causes of death were pancreatic fistula (2 cases), hemoperitoneum (2 cases, one of which was associated with a pancreatic fistula), dehiscence of the esophago-jejunal anastomosis (1 case), dehiscence of the duodenal stump (2 cases) and aspiration pneumonia resulting in ARDS (1 case). A higher mortality was observed in the group of patients aged > 65 years (7 cases out of 80, 8.7%) compared to those aged < 65 years (no cases in 106 patients, P = 0.002) and in N + patients (7 cases out of 112, 6.2%) compared to N- patients (no cases out of 74 patients, P = 0.04, Table 2).

In the univariate analysis a significant mortality rate was observed in the group of patients aged > 65 years (P = 0.008), in patients with Kattan score ≥ 100 points (P = 0.04), and in patients with advanced gastric cancer (P = 0.03). Sex (P = 0.4), type of surgery performed (P = 0.8), primary tumor location (P = 0.8), tumor depth (P = 0.1), and Lauren histological type (P = 0.4) had no statistically significant influence on

Table 1 Clinico-pathologic patient characteristics

Characteristics		
Sex		
Male	108	58.1%
Female	78	41.9%
Age, yr	64.9 ± 12.4	Range 24-90
Primary tumor location		
Gastroesophageal junction	22	11.8%
Upper third	25	13.4%
Middle third	58	31.2%
Lower third	81	43.5%
Histological type (Lauren classification)		
Enteric type	96	51.6%
Diffuse type	64	34.4%
Mixed type	26	14.0%
Type of resection		
Total gastrectomy	88	
Subtotal gastrectomy	98	
Size, cm	4.4 ± 2.3	Range 0.5-14
Number of lymph nodes retrieved	38.3 ± 10.9	Range 17-98
Number of positive lymph nodes	4.1 ± 3.6	Range 0-39
Operation time, minutes	260 ± 76.1	
Length of stay, d	12.7 ± 8.2	
T status		
T1a	36	19.3%
T1b	4	2.1%
T2	79	42.5%
T3	56	30.1%
T4a	2	1.1%
T4b	9	4.8%
Depth		
Mucosa	14	7.5%
Submucosa	31	16.7%
Muscularis propria	37	19.9%
Subserosa (suspected invasion)	46	24.7%
Subserosa (certain invasion)	49	26.3%
Serosa	4	2.1%
Adjacent structures	5	2.7%
N status		
N0	74	39.8%
N1	37	19.9%
N2	31	16.7%
N3a	33	17.7%

N3b	11	5.9%
Stage AJCC/TNM		
IA	29	15.6%
IB	32	17.2%
IIA	34	18.3%
IIB	35	18.8%
IIIA	23	12.4%
IIIB	26	14.0%
IIIC	7	3.8%

Values are mean ± SD. All the patients were included in all evaluations.

perioperative mortality (Table 3). In the multivariate analysis (Table 3) only age > 65 years had a statistically significant influence (T ratio 2.960, $P = 0.004$) on perioperative mortality.

Postoperative overall complications

Postoperative complications were documented in 40 patients (21.5%). Table 4 Lists the type of complications and their frequency. As shown, pulmonary complications, urinary tract infections, pancreatic fistulas, anastomotic leaks and duodenal fistula were the most frequently observed complications.

A higher incidence of complications was observed in patients undergoing subtotal gastrectomy (29 cases out of 98 patients, 29.5%) compared to those undergoing total gastrectomy (11 cases out of 88 patients, 12.5% - $P = 0.006$), in patients with Kattan score ≥ 100 points (32 cases out of 121 patients, 26.4%) compared to those with Kattan score < 100 points (8 out of 65 patients, 12.3% - $P = 0.02$) and in those N + (30 out of 112 patients, 26.7%) compared to those N- (10 of 74 patients, 13.5% - $P = 0.04$, Table 2).

Univariate analysis (Table 5) confirmed that sex, age, number of lymph nodes harvested, primary tumor site and histological type are not related to morbidity. This is related to the type of surgery ($P = 0.005$), the Kattan score ($P = 0.02$), the tumor depth ($P = 0.01$), T stage ($P = 0.006$) and the stage of the disease ($P = 0.01$). In the multivariate analysis (Table 5) only the extent of surgery showed a statistically significant correlation (T ratio 2.526, $P = 0.01$).

Postoperative surgical complications

Surgical complications were observed in 17 patients (9.1%). Among these, the most frequent were duodenal fistula (5 cases), pancreatic fistula (4 cases, one of which associated with hemoperitoneum) and dehiscence of the esophago-jejunal anastomosis. Four patients (2 cases of hemoperitoneum, 2 cases of duodenal fistula) underwent further surgical treatment. The two patients with bowel obstruction underwent adhesion lysis surgery 2 mo and 6 mo after gastric surgery, respectively. All other patients with surgical complications were treated conservatively. A higher incidence of surgical complications was observed in the patient group with more than 35 lymph nodes harvested (16 cases out of 103 patients, 15.5%) compared to patients in which fewer lymph nodes were removed (1 case in 83 patients, 1.2% - $P = 0.0005$). Sex ($P = 0.7$), age > 65 years ($P = 0.2$), type of surgery performed ($P = 0.6$), Kattan score ($P = 0.1$), lymph node positivity ($P = 0.1$) and early stage of disease ($P = 0.5$) did not affect the rate of perioperative surgical complications (Table 2).

This was confirmed by the univariate analysis, which documented that the removal of more than 35 lymph nodes ($P = 0.002$), the depth of the tumor ($P = 0.04$) and the stage of disease ($P = 0.01$) are statistically correlated with the development of surgical complications in the postoperative period (Table 6).

On multivariate analysis (Table 6) only one lymphadenectomy with removal of more than 35 lymph nodes correlates significantly with the rate of surgical complications (T ratio 3.222, $P = 0.001$).

Table 2 Mortality, overall morbidity and surgical morbidity in all patients

Characteristics	Number of cases	Mortality	Overall morbidity	Surgical morbidity
	186	7	40	17
Sex				
Male	108	3	24	9
Female	78	4	16	8
		$P = 0.4$	$P = 0.8$	$P = 0.7$
Age				
> 65 yr	80	7	22	10
≤ 65 yr	106	0	18	7
		$P = 0.002$	$P = 0.1$	$P = 0.2$
Type of surgery				
TG	88	3	11	7
STG	98	4	29	10
		$P = 1.0$	$P = 0.006$	$P = 0.6$
Kattan score				
≥ 100 points	121	7	32	14
< 100 points	65	0	8	3
		$P = 0.09$	$P = 0.02$	$P = 0.1$
Lymphadenectomy				
> 35 lymph nodes	103	3	24	16
≤ 35 lymph nodes	83	4	16	1
		$P = 0.7$	$P = 0.5$	$P = 0.0005$
Lymph nodes				
Negative	74	0	10	4
Positive	112	7	30	13
		$P = 0.04$	$P = 0.04$	$P = 0.1$
T				
Early cancer	40	0	5	2
Advanced cancer	146	7	35	15
		$P = 0.3$	$P = 0.1$	$P = 0.5$
Splenectomy	105	4	24	9
Spleen-preserving	81	3	16	8
		$P = 1.0$	$P = 0.7$	$P = 0.8$

TG: Total gastrectomy; STG: Subtotal gastrectomy. All the patients were included in all evaluations. Fisher exact test two-tailed.

DISCUSSION

Surgical treatment is still the mainstay of curative gastric cancer treatment[4,26-29]. For patients who undergo surgery, prognosis is determined by a series of factors, among which depth of invasion, nodal status, and metastasis are the most important. These factors are part of the UICC/AJCC stage formula, which is the most reliable prognostic system. In addition, certain multivariate analyses could identify extent of lymphadenectomy, lymph node ratio (ratio between positive and removed nodes), residual tumors, and grading, as independent prognostic factors. The expected prognosis has great impact on the kind of treatment a patient will receive. The

Table 3 Univariate and multivariate analysis of variables associated with postoperative mortality

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Sex (Male)	3	42.9	0.406	0.5888	0.557
Age > 65 yr	7	100	0.008	2.960	0.004
Type of surgery (subtotal gastrectomy)	4	57.1	0.810		
Kattan score ≥ 100	7	100	0.048	0.9504	0.343
Lymph nodes > 35	6	85.7	0.152	1.745	0.114
Primary site			0.821		
Gastroesophageal junction	1	14.3			
Upper third	1	14.3			
Middle third	1	14.3			
Lower third	4	57.1			
Depth			0.137	1.231	0.220
Mucosa	0				
Submucosa	1	14.3			
Muscularis Propria	0				
Subserosa (suspected invasion)	2	28.6			
Subserosa (certain invasion)	3	42.9			
Serosa	0				
Adjacent structures	1	14.3			
Histological type (Lauren classification)			0.436		
Enteric type	3	42.9			
Diffuse type	2	28.6			
Mixed type	2	28.6			
T status			0.031	1.342	0.181
T1a	0				
T1b	0				
T2	3	42.9			
T3	2	28.6			
T4a	0				
T4b	2	28.6			
Stage AJCC/TNM			0.039	0.6371	0.525
IA	0				
IB	0				
IIA	1	14.3			
IIB	2	28.6			
IIIA	1	14.3			
IIIB	3	42.9			
IIIC	0				

standard for nodal staging of gastric cancer has international variation, and recently significant changes have been made to the AJCC/UICC staging system to simplify lymph node staging in the countries using TNM staging. In the most recent AJCC edition N1 represents 1-6 positive lymph nodes; N2 represents 7-15 positive lymph

Table 4 Major postoperative complications with a severity grade 2 or more according Clavien-Dindo classification

Type of complication	Number of cases	%
Pulmonary	12	6.4
Urinary tract infection	10	5.4
Leak of esophago-jejunal anastomosis	4	2.1
Intra-abdominal abscess	1	0.5
Abdominal bleeding	2	1.0
Duodenal fistula	5	2.7
Intestinal occlusion	2	1.0
Pancreatic fistula	4	2.1

nodes; and N3 represents > 15 positive lymph nodes. The cut-off points were determined from retrospective databases[30] and in subsequent evaluations showed a superior predictive ability compared to other staging systems[31,32].

The extent of lymphadenectomy is the only factor that can be influenced by the surgeon[33-38]. The total number of lymph nodes resected, or the total number of positive to negative ratio of lymph nodes have all been found to be predictors of survival in gastric cancer patients[37]. For potentially resectable gastric cancer, a linear trend toward superior survival was found for higher lymph node removal up to 35-40 lymph nodes, based on the analysis of the SEER database from 1973 to 1999[38]. Adjuvant therapy is used in advanced gastric cancer to improve the survival and may be useful in high-risk patients treated with limited lymph node dissection. Moreover, lymph node dissection remains crucial to make every effort to improve the prognosis in those patients unsuitable for any adjuvant treatment[39,40]. In a study Biffi *et al*[13] showed that extended lymph node resection offers survival benefit even in the subgroup of patients with early-stage disease. Evaluation of distant disease-free survival risk by number of harvested lymph nodes showed that the risk of recurrence is inversely proportional to the number of dissected lymph nodes. The results did not change when pT1 and pT2-3 cancers were analyzed separately, suggesting the need to remove at least 15 nodes even in patients with early-stage disease[13].

The idea of an extended lymphadenectomy for gastric cancer was first advanced by Mikulicz in 1889, who stated that the distal pancreas should be removed if necessary [40-42]. Recent studies show that D2 lymphadenectomy improves the accuracy of locoregional staging and might reduce disease recurrence in patients with gastric adenocarcinoma[27]. Furthermore, when expert surgeons perform D2 lymphadenectomy and avoid routine distal pancreatectomy and splenectomy, perioperative morbidity and mortality can be kept to a minimum[43,44].

Although neither the 5-year[28] nor 11-year results[40] of the Dutch trial showed a significant improvement in overall survival for patients randomized to D2 lymphadenectomy compared with D1, we believe that surgery remains the only non-standardized therapy in the context of clinical trials and that D2 resection has clinical relevance in most treatment algorithms. Several surgeons agree that standardized D2 lymphadenectomy is an appropriate and potentially beneficial treatment approach[45,46]; like any therapy, surgery must be done safely and correctly by skilled clinicians and should be tailored to the patient and biology of the disease[4,47,48].

Marubini *et al*[10] examined 615 resections, and found no difference in mortality (1.8%) or complication rates (12.8%) with respect to the number of harvested nodes, but better overall survival when more lymph nodes were assessed. With more than 11 years of median follow-up, there was a trend for improved survival for patients with N2 disease who had received a D2 dissection[40]. Another analysis excluding patients with distal pancreatectomy and splenectomy found a survival benefit for the D2 resection patients[49]. Clinical series from Asia have found a low rate of nodal recurrences following aggressive lymph nodes dissection. Furthermore, Japanese investigators have recently completed trials of D2 *vs* D2 plus para-aortic nodal dissection, showing better results in small cancer with negative nodes who underwent aggressive D2 dissection[4]. Moreover, if D2 lymphadenectomy was performed, it was likely to have a marked benefit compared to D1 dissection[14,50].

Despite the therapeutic value of lymphadenectomy, mortality and complications are still high in gastric cancer surgery[16,51]. Several studies point out that stomach cancer

Table 5 Clinicopathological factors associated with overall morbidity by univariate and multivariate analysis

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Sex (Male)	24	60	0.779	0.8443	0.4
Age > 65 yr	22	55	0.575	0.4271	0.670
Type of surgery (subtotal gastrectomy)	29	72.5	0.005	2.526	0.012
Kattan score ≥ 100	32	80	0.026	0.5097	0.611
Lymph nodes > 35	24	60	0.962		
Primary site			0.180	0.3756	0.708
Gastroesophageal junction	5	12.5			
Upper third	1	2.5			
Middle third	7	17.5			
Lower third	27	67.5			
Depth			0.017	0.2270	0.821
Mucosa	1	2.5			
Submucosa	5	12.5			
Muscularis propria	5	12.5			
Subserosa (suspected invasion)	11	27.5			
Serosa	3	7.5			
Adjacent structures	4	10.0			
Histological type (Lauren classification)			0.265	0.4180	0.677
Enteric	17	42.5			
Diffuse	13	32.5			
Mixed	10	25.0			
T status			0.006	0.6177	0.538
1a	0				
1b	5	12.5			
2	15	37.5			
3	12	30.0			
4a	4	10.0			
4b	4	10.0			
Stage AJCC/TNM			0.018	0.8390	0.403
IA	5	12.5			
IB	2	5.0			
IIA	6	15.0			
IIB	10	25.0			
IIIA	7	17.5			
IIIB	8	20.0			
IIIC	2	5.0			

AJCC: American Joint Committee on Cancer.

surgery is a complex procedure that leads to a high risk of morbidity and mortality [15]. Li *et al*[52] observed 30 d and 90 d mortality of 2.0% and 3.4%, respectively, in patients undergoing total gastrectomy for cancer. These data are consistent with what

Table 6 Factors associated with surgical complications in univariate and multivariate analysis

	Univariate analysis			Multivariate analysis	
	Number of cases	%	*P* value	T ratio	*P* value
Male sex	9	52.9	0.653	0.4193	0.675
Age > 65 yr	10	58.8	0.502	1.192	0.235
Type of surgery (subtotal gastrectomy)	10	58.8	0.595		
Kattan score ≥ 100	14	82.3	0.116	0.08543	0.932
Lymph nodes > 35	16	94.1	0.002	3.222	0.001
Primary site			0.609		
Gastroesophageal junction	4	23.5			
Upper third	1	5.9			
Middle third	2	11.8			
Lower third	10	58.8			
Depth			0.045	0.8208	0.413
Mucosa	0				
Submucosa	2	11.8			
Muscularis Propria	2	11.8			
Subserosa (suspected invasion)	5	29.4			
Subserosa (certain invasion)	3	17.6			
Serosa	2	11.8			
Adjacent structures	3	17.6			
Histological type (Lauren classification)			0.817		
Enteric type	8	47.1			
Diffuse type	4	23.5			
Mixed type	5	29.4			
T status			0.054	1.102	0.272
T1a	0				
T1b	2	11.8			
T2	7	41.2			
T3	4	23.5			
T4a	1	5.9			
T4b	3	17.6			
Stage AJCC/TNM			0.019	0.8237	0.411
IA	1	5.9			
IB	0				
IIA	3	17.6			
IIB	5	29.4			
IIIA	3	17.6			
IIIB	4	23.5			
IIIC	1	5.9			

AJCC: American Joint Committee on Cancer.

is reported by other authors. Selby *et al*[53] reported data of 2.5% and 2.9% at 30 d and 90 d, respectively, while Pacelli *et al*[54] reported a mortality of 3.5% in 312 patients undergoing potentially curative gastrectomy for cancer. We observed a perioperative mortality rate of 3.8%. A higher mortality was observed in the group of patients aged > 65 years (8.7%) and in N + patients (6.2%).

The risk of postoperative complications is also high. Li *et al*[52] reports a complication rate of 43.9%, with a 14% incidence of severe (class III and class IV according to the Clavien-Dindo classification) complications. A severe complication after total gastrectomy is the anastomotic leak of the esophagojejunal anastomosis. In our experience, dehiscence occurred in 4 patients (2.1%), and was fatal in one case. Selby *et al*[53] and Pacelli *et al*[54] report an incidence of anastomotic dehiscence of 14.7% and 8.6% respectively. In our experience, all anastomotic leakages were identified in the early postoperative period, from day 4 to day 7, by performing routine upper GI contrast studies. The anastomotic leak leads to an increase in the duration of hospitalization, with increases ranging from 13 to 48 d of hospitalization [55]. Another severe complication is duodenal stump dehiscence. This complication occurred in 5 of our patients (2.7%), representing the cause of death in two of them. This complication also increased mortality in the literature[56]. We observed 2 cases of hemoperitoneum (1.0%) and 4 cases of pancreatic fistula (2.1%). These complications were fatal in the two cases of hemoperitoneum and in two of the 4 cases of pancreatic fistula. They were only observed in the patient group where more than 35 lymph nodes had been removed. In our series, mortality occurred only in the group of patients with a higher Kattan score. It seems likely that advanced stage tumors may alter the responsiveness of the patient, increasing the incidence of complications and mortality.

In our study, the overall incidence of surgery-related complications was 9.1%. As easy to predict, morbidity rate is higher in advanced tumors than in the earlier stage. The overall morbidity rate is higher in patients with Kattan score ≥ 100 (P = 0.02) and in N + patients (P = 0.04). Contrary to what has been observed in the literature, we documented a higher morbidity rate in patients undergoing subtotal gastrectomy (29 cases *vs* 11 cases after total gastrectomy - P = 0.006). We believe that this is related to a higher mean age in patients who underwent subtotal gastrectomy (66.6 ± 12.5 years, range: 24-90) than in those who underwent total gastrectomy (63 ± 12.1 years, range: 30-84, P = 0.04), and a higher mean Kattan score (125.1 ± 46.7 points, range 11-206) than in patients who underwent total gastrectomy (111.3 ± 44.1, range 24-215, P = 0.03). We observed a higher prevalence, without statistical significance (P = 0.2), of patients with Kattan ≥ 100 points in the group undergoing subtotal gastrectomy (64 patients, 65.3%) compared to those undergoing total gastrectomy (49 cases, 55.6%). Regarding other parameters considered, such as the size of the tumor (4.1 ± 2.0 cm in subtotal gastrectomy *vs* 4.6 ± 2.6 cm in total gastrectomy, P = 0.1), the average number of positive lymph nodes (3.5 ± 5.7 in subtotal gastrectomy *vs* 4.6 ± 2.6 in total gastrectomy, P = 0.1) we did not find statistically significant differences. The number of lymph nodes removed was higher in patients undergoing total gastrectomy (40 ± 10.4) than in those undergoing subtotal (36.7 ± 11.1, P = 0.03).

A higher incidence of surgical complications was observed in patients in whom more than 35 lymph nodes were removed. This data was confirmed in the univariate and multivariate analyses, where lymphadenectomy with the removal of more than 35 lymph nodes is the only factor that shows correlation with surgical complications. We have documented two cases of hemoperitoneum and 4 pancreatic fistulas, all in patients with spleen-preserving lymphadenectomy. Performing splenectomy for station 10 lymphadenectomy did not in our experience induce an increase in mortality and morbidity. These complications were found to be severe, as reported in the literature[26,57,58]. Many studies show that risk factors for the development of pancreatic fistula are the weight of the patient, the anatomy and texture of the pancreas, intraoperative trauma of the pancreas and the use of high-energy devices when performing lymphadenectomy[26,57].

Although we are aware that the Kattan nomogram was created to evaluate the long-term prognosis and survival of patients with gastric cancer undergoing R0 resection, we observed that the Kattan score, at the cut-off value used, is useful as a prognostic index even in the early postoperative phase. In our experience only patients with Kattan score ≥ 100 points died; a good correlation was also documented as far as the complication rate. Since Kattan takes into account, in addition to age, many characteristics of the tumor and the lymph node status, we have documented, as reported in the literature, that the incidence of mortality and major complications are observed with greater frequency in elderly patients, with more advanced and N + stage cancers. An intrinsic difficulty in using the Kattan score is the fact that the score itself is based on a

lot of histopathological information which are not always readily available.

All our patients underwent cholecystectomy. The procedure did not cause biliary complications. This aspect is controversial in the literature. In patients with a radical resection, when a D2 lymphadenectomy is performed and the duodenum is excluded in the intestinal reconstruction, cholecystectomy, considered by some to be a non-essential measure, is necessary to avoid gallstone formation and its complications. In this setting, we believe that prophylactic cholecystectomy is necessary for patients with a good cancer prognosis, as suggested by Pitt and Nakeeb[59]. Studies on the subject conclude that prophylactic cholecystectomy does not have a significant impact on the natural course of the disease[60]. However, it leads to a reduction in the number of biliary complications (which may affect up to 15% of the operated patients) and does not induce an increase in mortality and morbidity rates. In one study, a mortality rate of 1.8% was reported in the case of cholecystectomy performed during an intervention after a gastrectomy. Prophylactic cholecystectomy seems to be unnecessary only in cases where the continuity of the digestive tract involves the use of the duodenum[61]. It was found that the method used to restore intestinal continuity, with preservation of the duodenal transit or excluding the duodenum, is an independent risk factor for both the development of cholelithiasis (P = 0.018) and cholecystitis and cholangitis (P = 0.006). It has also been confirmed that in patients who develop cholelithiasis, the incidence of cholecystitis and cholangitis is particularly high when the duodenal transit is excluded (31.3%) compared to those with maintained duodenal transit (7.4%).

CONCLUSION

It is fair to reiterate that gastric cancer surgery is a complex surgical procedure. Mortality and postoperative complications are linked both to the extent of gastric demolition and to lymphadenectomy. In our experience, the removal of more than 35 lymph nodes conditioned an increase in surgical complications, although it did not lead to an increase in mortality. Mortality was higher in elderly patients, N + patients and patients with advanced gastric cancer. These parameters (age, T status and N status) are included in the Kattan score, which can be useful, if the histopathological parameters can be obtained quickly, as a prognostic tool even in the early phase.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer surgery is a complex procedure. Lymphadenectomy is essential for the surgical treatment of gastric cancer. Mortality and postoperative morbidity after gastric cancer surgery are not negligible.

Research motivation

We investigated in a population of 186 patients with stomach cancer undergoing surgery with D2 lymphadenectomy which factors were related to postoperative mortality and morbidity.

Research objectives

To evaluate the factors determining mortality and morbidity in a population of patients undergoing R0 resection and D2 lymphadenectomy for gastric cancer.

Research methods

For each patient we calculated the Kattan's score. The following prognostic variables were assembled for use in validating the nomogram: age, sex, primary site (distal one-third, middle one-third, proximal one-third, and gastroesophageal junction), Lauren histotype (diffuse, intestinal, mixed), number of positive lymph nodes resected, number of negative lymph nodes resected, and depth of invasion as defined by the standard nomenclature.

Research results

Perioperative mortality rate was 3.8% (7 patients); a higher mortality rate was observed in patients aged > 65 years (P = 0.002) and in N+ patients (P = 0.04).

Following univariate analysis, mortality was related to a Kattan's score ≥ 100 points (P = 0.04) and the presence of advanced gastric cancer (P = 0.03). Morbidity rate was 21.0% (40 patients). Surgical complications were observed in 17 patients (9.1%). A higher incidence of morbidity was observed in patients where more than 35 lymph nodes were harvested (P = 0.0005).

Research conclusions

Mortality and morbidity rate are higher in N+ and advanced gastric cancer patients. The removal of more than 35 lymph nodes does not lead to an increase in mortality.

Research perspectives

An extended lymph nodes dissection in patients undergoing surgical treatment for gastric cancer is a safe procedure.

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LETTER TO THE EDITOR

Prophylactic drains in totally laparoscopic distal gastrectomy: are they always necessary?

Tommaso Maria Manzia, Alessandro Parente, Roberta Angelico

ORCID number: Tommaso Maria Manzia 0000-0002-4636-3478; Alessandro Parente 0000-0001-5506-224X; Roberta Angelico 0000-0002-3439-7750.

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Tommaso Maria Manzia, Roberta Angelico, HPB and Transplant Unit, Department of Surgery Science, University of Rome Tor Vergata, Rome 00133, Italy

Alessandro Parente, Queen Elizabeth Hospital, Liver Unit, University of Birmingham, Birmingham UK; and HPB and Transplant Unit, Department of Surgery Science, University of Rome Tor Vergata, Rome 00133, Italy

Corresponding author: Tommaso Maria Manzia, FEBS, MD, PhD, Associate Professor, HPB and Transplant Unit, Department of Surgery Science, University of Rome Tor Vergata, Viale Oxford 81, Rome 00133, Italy. tomanzia@libero.it

Abstract

Prophylactic drains have always been a useful tool to detect early complications and prevent postoperative fluid collections, particularly in gastrointestinal surgery. Recently, the utilization of such drains has been debated, due to mounting evidence that they could be harmful rather than beneficial. Based on recent published articles, Liu *et al* reported that the routine use of prophylactic drains in total laparoscopic distal gastrectomy might not be necessary for all patients. Herein, we express our opinion regarding this interesting publication.

Key Words: Gastric cancer; Prophylactic drainage; Totally laparoscopic gastrectomy; Enhanced recovery after surgery; Minimally invasive surgery; Early gastric cancer

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Core Tip: Historically, prophylactic drains have been used to prevent postoperative collections and detect complications. In recent decades, there have been increasing reports that debate their routine usage in gastrointestinal surgery. Liu *et al* have shown that prophylactic drains can be safely omitted in selected patients undergoing totally laparoscopic distal gastrectomy. In this letter to the editor, we express our opinion regarding these interesting findings.

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TO THE EDITOR

We read with great interest the study by Liu *et al*[1]. These authors analyzed the outcome of 125 patients undergoing totally laparoscopic gastrectomy for distal gastric cancer with or without prophylactic drain (PD) insertion. In this retrospective study, Liu *et al*[1] demonstrated that in patients without placement of PDs there was no increased risk of postoperative complications. Furthermore, omitting a PD was associated with greater patient comfort. Of particular note, these interesting findings were confirmed by a propensity score matched analysis of 42 patients with and without PDs.

PDs facilitate the removal of postoperative fluid, which can potentially collect and become infected. In addition, PDs can help identify early postoperative complications such as anastomotic leakage and bleeding. In recent decades, the advances in surgical care have led to an overall decrease in postoperative complications. Therefore, the need for PDs has been debated and there is mounting evidence that they may even increase the risk of complications without preventing the need for reoperation. As an example, in major procedures such as liver resection, it has been shown that PDs increased the rate of biliary leak, length of hospital stay and total complications[2]. The same outcomes were demonstrated in gastric surgery in a recent meta-analysis[3].

This is one of the few studies which highlight the issues of PDs in total laparoscopic distal gastrectomy and, interestingly, the authors identified body mass index (BMI) ≥ 29 kg/m^2 to be associated with a higher risk of postoperative complications. The outcomes illustrated by the authors are in line with similar previously published articles[4,5]. Although the results by Liu *et al* are compelling, they need to be interpreted with caution. The data presented are prospectively maintained and retrospectively reviewed, but it is difficult to estimate the overall burden of postoperative morbidity as minor complications (Grade I), have not been included. Such examples are acute kidney injury treated with intravenous fluids, nausea treated with antiemetics, or electrolyte imbalances that responded to replacement therapy. This would add a more precise evaluation to the role of PDs in the postoperative setting, as minor complications could play an important role especially in the length of hospital stay. Secondly, the decision to insert a PD was made by the operating surgeon and the decision-making process which led to drain placement is unclear. This could bias the data, as it might be related to longer operative times and difficult surgery in high BMI patients. Thirdly, the cohort for this study was from a single-center, hence the generalizability to broader populations cannot be confirmed.

In summary, the authors should be commended for their work. They have demonstrated with a well-conducted analysis that PDs are not an independent risk factor for postoperative complications with the caveat that there appears to be a higher risk in patients with BMI ≥ 29 kg/m^2; therefore, in this group PDs are recommended. Identifying pre-operative and intraoperative factors that can guide the decision-making in order to select low-risk patients with regard to the omission of PDs would be of great interest. Furthermore, randomized controlled trials on PDs *vs* non-PDs insertion, focusing on laparoscopic approaches for gastric surgery, would be useful to guide clinical decisions.

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Contents

Weekly Volume 28 Number 4 January 28, 2022

FRONTIER

402 *Helicobacter pylori* infection: How does age influence the inflammatory pattern?

Araújo GRL, Marques HS, Santos MLC, da Silva FAF, da Brito BB, Correa Santos GL, de Melo FF

REVIEW

412 Gut bless you: The microbiota-gut-brain axis in irritable bowel syndrome

Hillestad EMR, van der Meeren A, Nagaraja BH, Bjørsvik BR, Haleem N, Benitez-Paez A, Sanz Y, Hausken T, Lied GA, Lundervold A, Berentsen B

432 Sarcopenia in hepatocellular carcinoma: Current knowledge and future directions

Perisetti A, Goyal H, Yendala R, Chandan S, Tharian B, Thandassery RB

MINIREVIEWS

449 Celiac disease: From genetics to epigenetics

Gnodi E, Meneveri R, Barisani D

ORIGINAL ARTICLE

Basic Study

464 Knockdown of DEAD-box 51 inhibits tumor growth of esophageal squamous cell carcinoma *via* the PI3K/AKT pathway

Hu DX, Sun QF, Xu L, Lu HD, Zhang F, Li ZM, Zhang MY

479 Fibrinogen-like protein 2 deficiency inhibits virus-induced fulminant hepatitis through abrogating inflammatory macrophage activation

Xiao F, Wang HW, Hu JJ, Tao R, Weng XX, Wang P, Wu D, Wang XJ, Yan WM, Xi D, Luo XP, Wan XY, Ning Q

LETTER TO THE EDITOR

497 Interplay between chronic hepatitis B and atherosclerosis: Innovative perspectives and theories

Ranković I, Milivojević V, Pavlović Marković A, Bezmarević M

500 Is CA19-9 effective in predicting chemotherapeutic response in patients with synchronous liver metastases with colorectal cancer?

Demirli Atici S, Kamer E

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Weekly Volume 28 Number 4 January 28, 2022

Contents

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FRONTIER

Helicobacter pylori infection: How does age influence the inflammatory pattern?

Glauber Rocha Lima Araújo, Hanna Santos Marques, Maria Luísa Cordeiro Santos, Filipe Antônio França da Silva, Breno Bittencourt da Brito, Gabriel Lima Correa Santos, Fabrício Freire de Melo

ORCID number: Glauber Rocha Lima Araújo 0000-0002-5195-3632; Hanna Santos Marques 0000-0001-5741-1570; Maria Luísa Cordeiro Santos 0000-0001-7078-9789; Filipe Antônio França da Silva 0000-0002-0550-1109; Breno Bittencourt de Brito 0000-0002-1831-7909; Gabriel Lima Correa Santos 0000-0003-3673-9889; Fabrício Freire de Melo 0000-0002-5680-2753.

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Glauber Rocha Lima Araújo, Maria Luísa Cordeiro Santos, Filipe Antônio França da Silva, Breno Bittencourt da Brito, Gabriel Lima Correa Santos, Fabrício Freire de Melo, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Vitória da Conquista 45029-094, Brazil

Hanna Santos Marques, Campus Vitória da Conquista, Universidade Estadual do Sudoeste da Bahia, Vitória da Conquista 45083-900, Brazil

Corresponding author: Fabrício Freire de Melo, PhD, Professor, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Rua Hormindo Barros, 58, Quadra 17, Lote 58, Vitória da Conquista 45029-094, Brazil. freiremelo@yahoo.com.br

Abstract

The inflammatory pattern during *Helicobacter pylori* (*H. pylori*) infection is changeable and complex. During childhood, it is possible to observe a predominantly regulatory response, evidenced by high concentrations of key cytokines for the maintenance of Treg responses such as TGF-β1 and IL-10, in addition to high expression of the transcription factor FOXP3. On the other hand, there is a predominance of cytokines associated with the Th1 and Th17 responses among *H. pylori*-positive adults. In the last few years, the participation of the Th17 response in the gastric inflammation against *H. pylori* infection has been highlighted due to the high levels of TGF-β1 and IL-17 found in this infectious scenario, and growing evidence has supported a close relationship between this immune response profile and unfavorable outcomes related to the infection. Moreover, this cytokine profile might play a pivotal role in the effectiveness of anti-*H. pylori* vaccines. It is evident that age is one of the main factors influencing the gastric inflammatory pattern during the infection with *H. pylori*, and understanding the immune response against the bacterium can assist in the development of alternative prophylactic and therapeutic strategies against the infection as well as in the comprehension of the pathogenesis of the outcomes related to that microorganism.

Key Words: *Helicobacter pylori*; Inflammation; Treg response; Th1 response; Th17 response; Gastric diseases

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Core Tip: *Helicobacter pylori* (*H. pylori*) is a bacterium acquired mainly in childhood that increases the risk of developing certain gastric diseases. However, the main complications are noticed predominantly in adulthood. This can be explained based on the gastric inflammatory pattern against the pathogen, which changes as long as the infected individual gets older, favoring, during childhood, the persistence of the infection and then, in adulthood, the gastric damage. This article discusses the factors that can influence the gastric inflammatory pattern in individuals infected with *H. pylori*.

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BIOGRAPHY

Fabrício Freire de Melo (Figure 1), PhD, is a professor at the Multidisciplinary Institute of Health of the Universidade Federal da Bahia, Brazil. He is currently a Research and Extension Coordinator at the aforementioned institute. He holds a bachelor's degree in Biological Sciences from the Pontifícia Universidade Católica de Minas Gerais (2004), in Brazil, and a master's degree (2007), a PhD (2011), and a postdoctoral fellowship (2013) in Biological Sciences (Microbiology) from the Universidade Federal de Minas Gerais (UFMG), Brazil. Moreover, he was a visiting researcher at the Medical Entomology Laboratory at the René Rachou Institute, Fiocruz, Brazil.

He divides his professional activity between research work and academic teaching. His main research areas include *Helicobacter pylori* infection, arboviruses, and, currently, *SARS-CoV-2*. He has developed extensive work on *Helicobacter pylori*, which includes investigations on the differences between the immune responses observed in children and adults infected with the bacterium. His work in several areas has already been recognized and awarded worldwide, being the cover of the *World Journal of Clinical Oncology* (Volume 11, Issue 11).

He is a member of the editorial board of the *World Journal of Clinical Oncology*, an academic editor of the *World Journal of Gastroenterology*, and a reviewer for journals including the *World Journal of Gastroenterology*, *World Journal of Clinical Cases*, and *World Journal of Gastrointestinal Oncology*.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative, rod-shaped, mobile bacterium of great clinical importance that is able to colonize the extremely hostile stomach environment[1].

Studies analyzing populations suggest that approximately 50% of the world population are infected with *H. pylori*. In addition, most *H. pylori* infections appear to be acquired during childhood, and estimates suggest that a third of the child population are or will be infected with the bacterium[2,3].

H. pylori infection is associated with the development of peptic ulcer and gastric cancer (GC), and the interactions between the virulence factors of the pathogen and the host immune response seem to be crucial in the development of those diseases[1,4]. Reviews show that 10% of those infected with *H. pylori* develop a peptic ulcer and 1%-3% develop GC[3]. *H. pylori* is a Group I carcinogen according to the International Agency for Research on Cancer (IARC), with 89% of all gastric cancers being attributable to this infection[2].

The host immune response to *H. pylori* is complex and changeable. It is possible to notice during childhood a predominantly regulatory inflammatory pattern (Treg), with higher concentrations of TGF-β1 and IL-10 than colonized adults, in addition to the greater number of FOXP3$^+$ Treg cells observed in the gastric mucosa of children. This predominantly regulatory pattern makes the gastric mucosa of children more



Figure 1 Fabrício Freire de Melo, PhD, Professor at the Universidade Federal da Bahia - Campus Anísio Teixeira, Brazil.

vulnerable to *H. pylori* colonization, but with milder inflammation when compared to what occurs in the mucosa of infected adults. As a result, the immune system of pediatric patients is not able to eliminate the *H. pylori* infection, and the bacterium persists in the gastric environment if left untreated. Moreover, damage to the gastric mucosa is less frequent during childhood, despite persistent mucosal colonization[5-8].

In adults, there is a predominant Th1 response, with higher levels of IFN-γ and IL-12p70 in the gastric mucosa, in contrast to the predominance of the regulatory response found during childhood. Besides, adults have a more intense Th17 response when compared to children. This can be verified by the higher mucosal concentrations of cytokines such as IL-17A and IL-23 and lower concentrations of TGF-β1, which, despite participating in the Treg response, when expressed at lower levels, seems to synergize with IL-6, promoting the expression of IL-23 receptors (IL-23r) and favoring an intense Th17 response. This cytokine profile is closely associated with the occurrence of damage to the gastric epithelium. Therefore, adults have a higher susceptibility to developing peptic ulcers, gastric atrophy, and intestinal metaplasia, a well-known precancerous lesion[5,6,9].

Of note, an increase in pro-inflammatory cytokines such as TNFα, IL-1α, IL-1β, IL-6, IL-2, and IL-17A is observed in *H. pylori*-positive children compared to *H. pylori*-negative infants. However, the Treg profile seems to overcome the inflammatory responses promoted by Th1 and Th17 cytokines in those individuals. This predominance of a regulatory immune response observed in infants might favor the colonization and persistence of the infection in the gastric mucosa, whereas the Th1 and Th17 responses induce a higher inflammatory activity in adults, leading to a higher risk of *H. pylori*-related gastric damage.

PREVALENCE

H. pylori infect about 4.4 billion people worldwide[2]. The prevalence of the infection is variable around the world and has changed over the last few years, with a notable reduction of the *H. pylori*-infected population, especially in developed countries[2,10-12]. Hooi *et al*[2] showed, through a meta-analysis, that the seroprevalence is higher in underdeveloped regions, and the highest prevalences were found in Africa (79.1%), Latin America and the Caribbean (63.4%), and Asia (54.7%). Otherwise, developed regions such as North America (37.1%) and Oceania (24.4%) have lower prevalence rates[3].

The infection is mainly acquired during childhood, and this phenomenon is predominantly observed in countries with a higher prevalence of *H. pylori*-positive individuals[13-15]. Moreover, higher prevalences of *H. pylori* infection are associated with lower socioeconomic status, household overcrowding, and lower educational

levels[11]. Sex may also influence the risk of acquiring the infection. A higher prevalence of the disease is usually observed among male subjects than in females. This may be related to hormonal factors, especially estrogen, which stimulates the immune response, and to a lower exposure to environmental factors such as smoking among women[16,17].

Furthermore, the prevalence may vary based on ethnic groups: Indigenous people in most countries are more susceptible to being infected[2]; a study in the United Arab Emirates showed a higher *H. pylori* prevalence among Africans than in Asian and Arabic populations, and, despite living in similar conditions to other ethnic groups, Malays were notably less affected by *H. pylori* infection than other people in that country[18-20]. In another study, Jonaityte *et al*[21] found a decline in the seroprevalence of *H. pylori* among medical students from Lithuania, with seroprevalences of 51.7, 30.4, 26.3, and 14.2% in 1995, 2012, 2016, and 2020, respectively. Besides, Africa, Western Asia, and South America are the regions with the highest incidence of *H. pylori*, while Oceania, North America, and Western Europe have lower prevalences of the bacterium[2].

BACTERIAL DENSITY AND GASTRIC INFLAMMATION

Despite being able to colonize all regions of the stomach, *H. pylori* proliferates better in certain anatomical areas, and higher bacterial densities are found in the antrum and cardia. Many factors can be responsible for this difference, such as the different levels of acid production in each portion of the stomach. In this sense, the regions with slightly lower acidity (antrum and cardia) are the regions with the highest *H. pylori* density[22,23].

Margarida *et al*[24], when studying 21 children infected with *H. pylori*, found infiltration of mononuclear (MN) cells in 50% of the cases. Furthermore, they did not find any neutrophil infiltrate in 40% of the participants, and, in 60% of the individuals, there was a slight eosinophilic infiltrate. Moreover, they have also found a relationship between bacterial density and MN and neutrophil cell counts in the stomach. Besides, they concluded that the infiltration of MN cells and neutrophils is lower in children infected with *H. pylori* than in *H. pylori*-negative adults. These findings were probably due to the differences between the immune response profiles predominating in each age group[2,9]. Thus, it is evident that the host immune response directed to the *H. pylori* can be influenced by several factors such as age and bacterial density, being complex and changeable.

CYTOKINE CONCENTRATIONS IN THE GASTRIC MUCOSA OF CHILDREN AND ADULTS

Given that *H. pylori* colonization is established mainly during childhood, that severer clinical outcomes related to the infection tend to occur as age advances, and that the immune system plays pivotal roles in *H. pylori*-related diseases, the following question is raised: Is the cytokine pattern observed in the immune response against the bacterium influenced by the age of colonized patients?

In an investigation enrolling Brazilian children and adults, our group has demonstrated that, among *H. pylori*-infected persons, infants tend to present a gastric Treg-polarized cytokine profile instead of the significant expression of Th17-related cytokines observed in older individuals. The analysis of the expression of cytokines in the gastric environment evidenced that IL-10 and TGF-β1 are expressed at higher levels in the former group, whereas the contrary was observed regarding the expression of IL-1β, IL-17A, and IL-23[5]. Those findings corroborate a precedent study by Harris *et al*, which showed more intense expression of the Treg-related cytokines TGF-β1 and IL-10 in children than in adults in a Chilean population[8]. Another study carried out by our group evaluated cytokines associated with innate and Th1 immune response in *H. pylori*-positive patients from various age groups[9]. We found that the gastric levels of IL-1α and TNF-α were significantly higher in children than in adults, whereas IL-2, IL-12p70, and IFN-γ were less expressed in infants than in older individuals (Figure 2). Interestingly, a drop in the gastric concentrations of IFN-γ and IL-12p70 in adults and an increase in the gastric mucosa levels of IL-1, IL-2, IL-12p70, and IFN-γ in children were observed with aging.

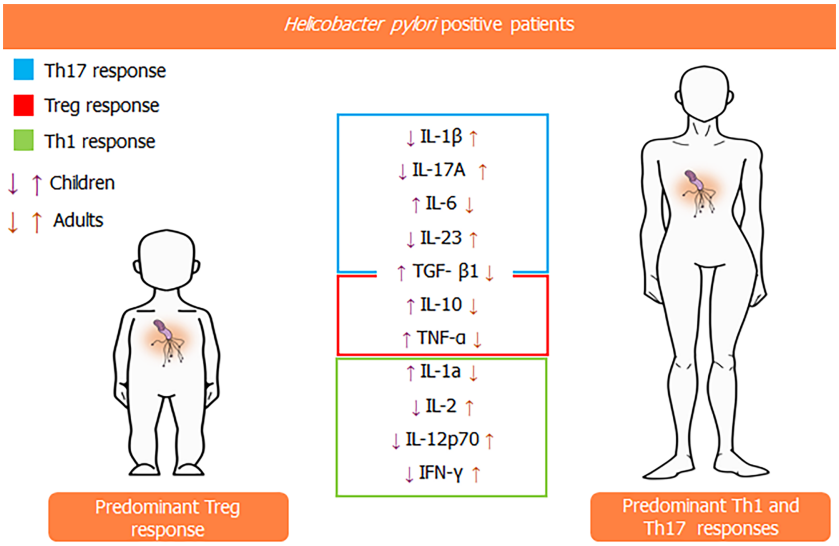


Figure 2 Comparison between gastric cytokines levels in children and adults.

Taken together, the aforementioned results show that age, indeed, influences the immune response against the bacterium and strongly suggest the occurrence of significant anti-inflammatory patterns among *H. pylori*-infected children, which might affect not only the development of gastric diseases but also other health-related aspects during the initial years of life. This hypothesis becomes even more relevant when considering that environmental stimuli are crucial for the development of the immune system in that life period, a position supported by the so-called hygiene hypothesis, which claims that the contact with microorganisms in early life is determinant for the maturation of the immune system[25]. Interestingly, a recent study by León *et al*[26] suggests that *H. pylori* may induce atopy modulation in children since they found that *H. pylori*-infected infants had higher expression of high-affinity IgE receptor (FcεRI) by peripheral dendritic cells and enhanced levels of FOXP3 and Latency Associated Peptide by T reg cells. The FcεRI is related to a regulatory dendritic cell profile since the interaction of IgE with that molecule fails to induce the maturation of these cells [27]. The possibility of systemic effects by *H. pylori* infection through the induction of immune system regulatory mechanisms makes us question the possible impacts of *H. pylori* eradication among children over the development of future immune system-related disorders. Although we understand and support the need for eliminating the bacterium, it has to be emphasized that this infection has been negatively correlated to the development of relevant immune system-linked diseases that are relevant among young people, including asthma[28]. In addition, the current scenario of widespread use of antibiotics and growing antimicrobial resistance among *H. pylori* strains should not be ignored[29]. Therefore, we hope that, along with the advances in the clinical analysis of genetic and epigenetic backgrounds, the future approaches to *H. pylori* infections and the decision on the necessity of bacterial eradication should be carried out in a more individualized manner, instead of the generalized, but necessary, treatments preconized by current guidelines.

Some studies have emphasized that immunizing agents against *H. pylori* should be able to induce a Th17 response to achieve satisfactory effectiveness. In that context, Velin *et al* induced mouse immunization using mucosally administered cholera toxin followed by *H. felis* challenge and observed that it induced a remarkable peak of CD4+IL-17+ T cells in the gastric mucosa[30]. Recently, a study by Chen *et al*[31] tried to immunize mice using a cyclic guanosine monophosphate-adenosine monophosphate as an adjuvant for the anti-*H. pylori* vaccine and observed that its effectiveness depended on high levels of antigen-specific Th1 and, mainly, Th17 responses. These findings draw attention to the aforementioned results showing low levels of Th17-related cytokines among *H. pylori*-infected children, which could represent an obstacle in the development of effective immunizing agents for that population. This is an important issue to be considered since the *H. pylori* infection is mainly acquired during childhood[31].

GASTRIC HISTOLOGY AND CYTOKINE CONCENTRATIONS

In our aforementioned study evaluating the variations of the Th1 immune response to the infection by *H. pylori* according to age, we observed that the increased levels of IFN-γ and IL-12p70 in the gastric environment were associated with an increase in MN cells in the gastric corpus and antrum. Moreover, when considering the group of young adults, IL-12p70 was linked to an increase in the count of both MN and PMN cells in the gastric antrum[9]. Interestingly, another study observed that the levels of IFN-γ and IL-12 were higher in infected children than in uninfected children ($P < 0.001$). In addition, these cytokines were positively correlated with the inflammation score ($P < 0.01$) and PMN infiltration, corroborating our findings[32]. In an analysis of polyclonal responses in $CD4^+$ T cells in *H. pylori*-positive children, a potent production of IFN-γ was also observed. However, the responses were stronger in adults, due to their higher frequency of memory T cells[33]. Curiously, some authors have observed that the levels of *IFN-γ* mRNA in infected children were lower when compared to infected adults[8,34]. These data suggest an increased regulatory response conducted by Treg cells in children, thus reducing the inflammatory Th1 response in the gastric mucosa[5,8]. In a recent prospective Brazilian study, it was observed that IL-27 is increased in individuals with *H. pylori*-related duodenal ulcer and absent in patients with GC. Moreover, higher gastric concentrations of IL-12p70 ($P < 0.001$) and IFN-γ ($P = 0.004$) were observed in patients with duodenal ulcers than in those with GC. In addition, IL-27 is positively correlated to the expression of IL-12p70, an important cytokine in Th1 responses that directly influences the pattern of inflammation in the antral mucosa of patients with duodenal ulcer[35]. The relationship between IL-12p70 and IFN-γ is well elucidated in the context of *H. pylori* infection. In a study that added neutralizing antibodies to IL-12 in gastric biopsy cultures, authors observed a negative regulation of signal transducer and activator of transcription 4 (STAT4), an important factor for the production of IFN-γ, leading to a significant decrease in the concentrations of this cytokine ($P < 0.001$)[36]. Therefore, considerable progress has been achieved in the understanding of these important interplays between cytokine variations between different age groups and among regions of the gastric mucosa. Although the presence of MN and PMN cell infiltration associated with Th1 responses has been described, further studies are needed to aid in the understanding of the dynamics and frequency of these cells in the context of the *H. pylori*-induced gastric diseases.

As aforementioned, the *H. pylori* gastric environment colonization leads to a polarization toward Th1/Th17 responses, whereas Treg cells are responsible for the induction of anti-inflammatory responses. Of note, the Treg cells can be divided into IL-10-secreting Tr1 cells, TGF-β1-producing Tr3 cells, and FOXP3-expressing CD4+CD25high Treg cells[37]. The latter cells seem to be crucial in the setting of *H. pylori* infection. As long as they suppress the immune response against the bacterium, the pathogen persistence in the gastric mucosa might be favored. In that context, when evaluating the host immune response against *H. pylori* in adults and children, our group found that the expression of $FOXP3^+$ Treg cells was significantly higher in the antrum of *H. pylori*-positive patients than in *H. pylori*-negative individuals[38]. This finding corroborates a previous study by Kandulski *et al*[39], which reported that *H. pylori* infection leads to a remarkable proportional enhancement of $FOXP3^+$ Treg cells in the gastric cardia and antrum. In addition, the study by Silva *et al*[40], in its turn, reported that the levels of FOXP3-positive cells depend on the presence of gastritis. They observed that individuals with active chronic gastritis have lower expression of this molecule than persons without gastritis. Against this background, it is possible to infer that those cells are crucial for the occurrence of *H. pylori*-related diseases since they are directly associated with the levels of gastric mucosa inflammation.

Another finding in our study was the significantly higher levels of Treg $FOXP3^+$ cells in children than in adults in the setting of *H. pylori* infection. Along with the cytokine pattern in pediatric patients previously discussed in this paper, this data indicates a milder infection with the bacterium in infants than in older individuals. Furthermore, a recent investigation using animals observed that mice infected during the neonatal period are more intensely colonized with the bacterium than those infected during adulthood. The neonatally infected mice had an immune response characterized by an intense infiltration of $FOXP3^+$ Treg cells, and this result was VacA-dependent. Moreover, the study identified that the presence of VacA led to enhanced expression of IL-10 and TGF-β in macrophages whereas it suppressed the production of IL-23 in dendritic cells[41]. Another subsequent study by Altobelli *et al*[41] corroborates our hypothesis that the younger the host, the milder the inflammatory response against the bacterium with increased levels of $FOXP3^+$ Treg cells. They used mice to

evaluate the role of the induction of the co-inhibitory receptor B7-H1 in the chronic *H. pylori* infection and demonstrated that the induction of the Treg profile as well as the inhibition of T cell proliferation and IL-2 production are mediated by the B7-H1 expression, which results from the *H. pylori* type 4 secretion system (T4SS) action through the activation of the p38 MAPK pathway[42,43]. Interestingly, a recent study reported that animals infected with the *H. pylori* PMSS1 strain had higher levels of Treg cells and lower levels of Th17 cells than animals infected with the SS1 *H. pylori* strain[44]. Taken together, these studies show the plurality of factors influencing the induction of Treg cells in the gastric environment of *H. pylori*-positive individuals.

Notably, Wei *et al*[45] suggested that the immune response against *H. pylori* characterized by the expression of Treg FOXP3$^+$ cells and IL-10 is not only observed in the gastric mucosa, but it is also enhanced in both superior and inferior gastrointestinal tracts after 10 wk of infection, suggesting a systemic character of this regulatory immune response. Data from another study identified significant enhancement of FOXP3 expression in patients with MALT lymphoma compared to individuals with active chronic gastritis. Interestingly, *H. pylori*-positive MALT lymphoma patients with increased expression of Treg FOXP3$^+$ cells were significantly more responsive to the *H. pylori* eradication therapy than those with lower expression of Treg FOXP3$^+$ cells[46]. In addition, Sen *et al*[47] reported significant enhancement in the levels of FOXP3 expressed by T CD25+ CD127 low/- cells in the peripheral blood of patients with GC compared to the control group, and the T CD25+ CD127 low/- cells were also present in the tumor microenvironment and contributed to the suppression of T effector cells against the tumor. These results suggest a relevant role of the *H. pylori*-induced immune system regulation by FOXP3-expressing cells in the scenario of the development and progression of malignancies associated with *H. pylori* gastric infection.

Finally, we demonstrated that children infected with *H. pylori* had Treg FOXP3 cell levels positively correlated with IL-10 expression in the gastric antrum and negatively correlated with the count of mononuclear and polymorphonuclear cells. Moreover, the levels of FOXP3$^+$ Treg cells were also negatively correlated with mononuclear cells in adults. In that context, Gil *et al*[4] evaluated the expression of FOXP3, IL-10, TGF, and IL-17A as well as the dynamics of Th17/Treg FOXP3$^+$ cells in the gastric mucosa of *H. pylori*-positive children. Their data showed that FOXP3, TGF-β1, and IL-10 were remarkably expressed in the infection and the number of FOXP3$^+$ Treg cells was significantly enhanced among *H. pylori*-positive individuals compared to *H. pylori*-negatives. Moreover, FOXP3 was positively related to the bacterial density as well as with the number of polymorphonuclear and mononuclear cells among *H. pylori*-positive persons with gastritis. Therefore, the data provided by Gil *et al*[4] reinforce the influence of FOXP3 expression in the control of *H. pylori*-induced gastric inflammation and in the recruitment of mononuclear and polymorphonuclear cells, important components of the immune response against the pathogen and in the pathogenesis of diseases associated with this infection.

CONCLUSION

H. pylori infection remains an important determinant for gastric illness. Several factors can alter the host inflammation pattern directed to the bacterium, and it is evident that age is one of the most important variables in that setting. A better understanding of the immune system behavior at different ages, favoring, during childhood, the persistence of the infection and then, in adulthood, the gastric damage, can aid in the development of strategies aiming at the reduction of *H. pylori* prevalence, such as vaccines, and at the prevention of unfavorable infection-related clinical outcomes.

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REVIEW

Gut bless you: The microbiota-gut-brain axis in irritable bowel syndrome

Eline Margrete Randulff Hillestad, Aina van der Meeren, Bharat Halandur Nagaraja, Ben René Bjørsvik, Noman Haleem, Alfonso Benitez-Paez, Yolanda Sanz, Trygve Hausken, Gülen Arslan Lied, Arvid Lundervold, Birgitte Berentsen

ORCID number: Eline Margrete Randulff Hillestad 0000-0002-4155-294X; Aina van der Meeren 0000-0001-6181-0929; Bharat Halandur Nagaraja 0000-0002-5758-3097; Ben René Bjørsvik 0000-0002-7883-0405; Noman Haleem 0000-0001-7588-6166; Alfonso Benitez-Paez 0000-0001-5707-4340; Yolanda Sanz 0000-0002-1615-1976; Trygve Hausken 0000-0001-7080-8396; Gülen Arslan Lied 0000-0002-1827-5008; Arvid Lundervold 0000-0002-0032-4182; Birgitte Berentsen 0000-0003-3574-7078.

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Eline Margrete Randulff Hillestad, Trygve Hausken, Birgitte Berentsen, Department of Clinical Medicine, University of Bergen, Bergen 5021, Norway

Eline Margrete Randulff Hillestad, Aina van der Meeren, Ben René Bjørsvik, Noman Haleem, Trygve Hausken, Gülen Arslan Lied, Birgitte Berentsen, National Center for Functional Gastrointestinal Disorders, Department of Medicine, Haukeland University Hospital, Bergen 5021, Norway

Bharat Halandur Nagaraja, Ben René Bjørsvik, Noman Haleem, Arvid Lundervold, Mohn Medical Imaging and Visualization Center, Department of Radiology, Haukeland University Hospital, Bergen 5021, Norway

Alfonso Benitez-Paez, Host-Microbe Interactions in Metabolic Health Laboratory, Principe Felipe Research Center, Valencia 46012, Spain

Yolanda Sanz, Microbial Ecology, Nutrition and Health Research Unit, Institute of Agrochemistry and Food Technology, National Research Council, Paterna-Valencia 46980, Spain

Gülen Arslan Lied, Center for Nutrition, Department of Clinical Medicine, University of Bergen, Bergen 5021, Norway

Arvid Lundervold, Department of Biomedicine, University of Bergen, Bergen 5021, Norway

Corresponding author: Birgitte Berentsen, MSc, PhD, Associate Professor, National Center for Functional Gastrointestinal Disorders, Department of Medicine, Haukeland University Hospital, Jonas Lies vei 65, Bergen 5021, Norway. birgitte.berentsen1@helse-bergen.no

Abstract

Irritable bowel syndrome (IBS) is a common clinical label for medically unexplained gastrointestinal symptoms, recently described as a disturbance of the microbiota-gut-brain axis. Despite decades of research, the pathophysiology of this highly heterogeneous disorder remains elusive. However, a dramatic change in the understanding of the underlying pathophysiological mechanisms surfaced when the importance of gut microbiota protruded the scientific picture. Are we getting any closer to understanding IBS' etiology, or are we drowning in unspecific, conflicting data because we possess limited tools to unravel the cluster

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of secrets our gut microbiota is concealing? In this comprehensive review we are discussing some of the major important features of IBS and their interaction with gut microbiota, clinical microbiota-altering treatment such as the low FODMAP diet and fecal microbiota transplantation, neuroimaging and methods in microbiota analyses, and current and future challenges with big data analysis in IBS.

Key Words: Microbiota; Neurogastroenterology; Irritable bowel syndrome; Microbiota-gut-brain axis; Structural and functional magnetic resonance imaging; Machine learning; Big data analysis

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Core Tip: Molecular biology, advanced neuroimaging and computer science is emerging to transform our understanding of the role of gut microbiota in irritable bowel syndrome (IBS). Herein, we provide an overview and discuss the role of gut microbiota in IBS, the clinical microbiota-altering interventions the low FODMAP diet and fecal microbiota transplantation, the role of brain-imaging and gut microbiota analyses, the importance of method selection, metadata, perspectives for improving microbiota role predictions, and big data analysis, in the seeking of understanding IBS pathology.

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INTRODUCTION

Irritable bowel syndrome – a disturbance of the microbiota-gut-brain axis

Irritable bowel syndrome (IBS) is a chronic biopsychosocial disorder manifested by recurrent abdominal pain and alterations in stool form or frequency[1]. The condition affects 4%-10% of the global population and is associated with markedly reduced quality of life[2,3]. In addition to genetic predisposition, adverse life events, psychosocial factors, chronic and acute stress, and gastrointestinal (GI) infections[1], mounting evidence suggests the gut microbiota play a key role in IBS.

Because of its heterogeneity and unclear etiology, clear biomarkers and therapeutic targets for IBS have been difficult to identify. As a term, "IBS" is collective for medically unexplained disturbances of the bidirectional communication between the gut and the brain. These disturbances are multifactorial and include visceral hypersensitivity, low-grade inflammatory responses, intestinal motility disturbances, alterations of central nervous system (CNS) processing, and alterations in gut microbiota composition[1]. In the gut, a well-functioning microbiota is highly adapted to the host and carries out biochemical and metabolic processes that are important for host function. Signals coming from the gut microbiota modulate aspects of homeostasis through neural, endocrine and immune communication pathways between the gut and the brain[4,5]. Together, this has established the concept of the microbiota-gut-brain (MGB) –axis (Figure 1).

The vagus nerve serve as a major MGB pathway modulator. It is composed of somatic and afferent fibers (80%) and general and special visceral efferent fibers (20%). Under normal circumstances, the vagus nerve sense and is activated by diet-responsive gut microbes and metabolites such as short-chain fatty acids (SCFAs), or endocrine factors, enzymes, and neurotransmitters such as serotonin, dopamine, acetylcholine, glutamate, γ-aminobutyric acid (GABA), and noradrenaline[6-9]. Each of these factors are potentially affected by alterations in microbiota composition and are involved in IBS pathology, as shown in Figure 1. In the intestines, vagal endings synapse onto neurons of the enteric nervous system (ENS), which governs the function

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of muscular, neuro-hormonal, and secretory systems of the GI tract to generate patterns of functional digestion. In IBS, the pathophysiology implicates altered gut microbiota composition, impaired intestinal mucosal integrity, and low-grade inflammation[10]. In addition to pathways through the circulatory system, several of these factors may also trigger fluctuations in the activity of the ENS with subsequent effect on the brain. This relationship of reciprocal signals may be disturbed to the degree of chronic IBS. In the chronic IBS brain, efferent signals may be perceived as unpleasant or painful, potentially leading to chronic visceral discomfort or pain[11].

The heterogeneity of the "healthy gut microbiota" has made it difficult to identify a clear IBS microbial signature. Indeed, the composition of gut microbiota composition is influenced by multiple factors, *e.g.*, geographic location, ethnicity, dietary choices, medication use, and pathogens, summarized by Adak *et al*[12]. Hence, the human gut microbiota composition is highly diverse. This heterogeneity makes is difficult to provide a clear definition to what a "healthy microbiota" is. Nevertheless, some features are considered important characteristics: a high level of diversity, a favorable amount of butyrate-producing bacteria, and resistance and resilience - the ability to withstand a disturbance promoting a shift in the composition and the attribute to return to its initial composition, functionally or taxonomical, following this disturbance[12,13]. On the contrary, in disease the microbiota composition is often associated with a decreased microbial diversity and loss of the typical balance between the host and the microorganisms[13], a so-called "dysbiosis" (a debated concept[14,15]), linked to several systemic and local human diseases.

Multiple studies have shown differences in the gut microbiota between IBS and healthy controls[16-20]. A comprehensive systematic review from 2019 showed that patients with IBS have increased levels of the bacterial families Enterobacteriaceae, Lactobacillaceae and Bacteroidales, whereas *Bifidobacterium*, *Faecalibacterium*, and Clostridiales were decreased compared to healthy controls[21]. On the contrary, Hugerth *et al*[22] recently reported no distinct microbiota signature of IBS in a random Swedish population of 3556 participants. Here, the between-sample divergence was higher in IBS compared to controls from the same population-sampling frame, but no clear biomarker of IBS was revealed. There are multiple individual reports on differences in distribution patterns of constipation predominant IBS (IBS-C), IBS with mixed constipation and diarrhea (IBS-M), and diarrhea predominant IBS (IBS-D), summarized by, among others, Liu *et al*[23], and Wang *et al*[24]. Pozuelo *et al*[25] found butyrate- and methane-producing bacteria were less abundant in IBS-D and IBS-M patients. However, Pittayanon *et al*[21] summarized six studies from 130 patients with IBS-M, demonstrating no significant difference between subtypes. Interestingly, intestinal bacterial composition has been reported to be highly dependent on sample type and regional localization. Also, mucosa-associated bacterial composition of the sigmoid colon differ between patients with IBS and healthy controls[26].

Indeed, the absence of a universal definition of what a "healthy microbiota" is, in addition to lack of consistency in sequencing methodology, study protocols, inter-individual variation that dominate intra-individual variation, definitions of "controls", and different statistical methodologies being used have made the search for a common pathological IBS microbiota signature difficult. The importance of method selection, metadata, and perspectives for improving microbiota role predictions are discussed more thoroughly in section "Intestinal microbiota analyses", below.

Another factor to consider is the impact of the circadian rhythm on gut microbiota variability. Both the level of host-derived autoantibodies and peptides and nutrient availability give fluctuations in the gut microbiota, and both are associated with circadian rhythm oscillations[6]. At least 10% of operational taxonomic units may oscillate due to the circadian rhythm, which is important to consider when collecting and analyzing fecal samples[27]. Thus, we might be in the mere beginning of understanding how alterations in gut microbiota may lead to the disruption of the intricate host-gut-microbiota-interaction. Is it a cause or a result of IBS pathology? In the last decade, much knowledge has been gained from clinical microbiota-altering interventions such as the low FODMAP-diet and fecal microbiota transplantation (FMT), which has emerged as debatably successful treatment strategies. However, their effects on the MGB-axis are still far from understood.

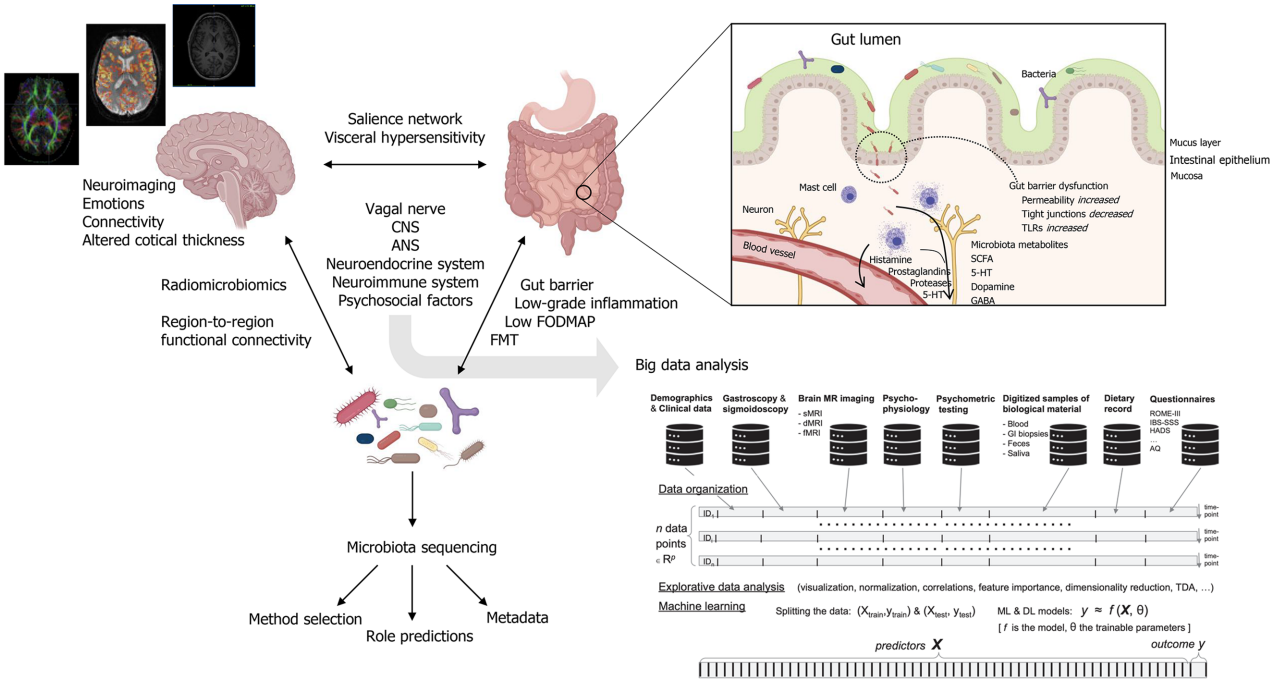


Figure 1 Integration of multimodal and interdisciplinary approaches for big data analysis in irritable bowel syndrome. Created with BioRender.com. ANS: Autonomic nervous system; CNS: Central nervous system; FODMAP: Fermentable oligosaccharides, disaccharides, monosaccharides and polyols; FMT: Fecal microbiota transplant; 5-HT: 5-hydroxytryptamine (serotonin); SCFAs: Short chain fatty acids; GABA: γ-aminobutyric acid; TLRs: Toll-like receptors.

CLINICAL MICROBIOTA-ALTERING TREATMENT IN IBS – THE LOW FODMAP DIET AND FECAL TRANSPLANTATION

Dietary intervention

Diet is an environmental factor that is pivotal in shaping the architecture of gut bacteria. Although genetics have been assumed to be of great importance[28], a recent study shows that environmental factors, such as diet, are dominating[29]. In the symbiotic host/bacteria relationship, gut bacteria depend on host intake of complex polysaccharides to facilitate growth. As hosts, humans depend on gut bacteria to break down complex nutrients resistant to human GI metabolism and metabolites produced from fermentation, such as SCFAs. In IBS, foods play an important role among the contributing factors to symptom induction. In fact, the majority of patients with IBS experience increased symptom burden after food intake[30], despite the lack of objective evidence for food hypersensitivity or allergies[31]. Several underlying mechanisms generating symptoms are proposed to be involved[32]: (1) Local effects in the small and large intestine are caused by fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs). Intake of these short-chain carbohydrates have an osmotic effect in the gut lumen, increasing small intestinal water content and introduce undigested food particles to the gut bacteria who readily ferment them, causing gas production in the colon leading to abdominal pain as a consequence of a sensitive ENS i.e.: visceral hypersensitivity[33]. In 2017, Varjú *et al*[34] compared standard dietary therapy for IBS and the low FODMAP diet in 2017. Both diets showed to alleviate symptoms, but the low FODMAP diet showed a better therapeutic effect. However, with the available data on diet interventions in IBS, a low FODMAP diet has the greatest evidence of efficacy[35], with the most updated systematic review reporting significant improvements in GI symptoms and quality of life compared to control diets or habitual diets[36]; (2) Gut microbiota alterations and bacterial fermentation might have a role in food-related symptoms. Inexplicably, contradicting findings regarding different microbiota compositions between patients with IBS and healthy controls are often reported[21,22]. A recent matched case-control study from a Thai population reported no distinction in the gut microbiota between IBS-D and healthy subjects[37]. Here, the authors accredit the discordant results from those conducted in Western countries as an effect of different dietary lifestyles affecting the gut microbiota, suggesting that alterations in gut microbiota is not the main pathogenic mechanism of IBS-D in Thai patients[37]. In the Swedish random

population, patients with IBS showed higher heterogeneity in microbiota composition compared to healthy individuals[22]. However, we need to keep in mind that bacterial fermentation capability may be more dependent on bacterial function rather than composition alone[38]. Nevertheless, differences in composition may still matter because it could result in differences in the effectiveness of a function[39]. Despite good documentation of a low FODMAP diet on symptom alleviation[36], FODMAP restriction is of concern due to possible unhealthy changes in gut microbiota composition with unknown consequences. Depriving the gut bacteria of carbohydrate and prebiotic substrates will shift the gut microbiota to ferment *e.g.*, proteins and/or some amino acids, leading to production of potentially harmful compounds, summarized by Oliphant and Allen-Vercoe[40]. Desai *et al*[41] investigated gnotobiotic mice colonized with human gut microbiota fed a fiber-deprived diet. Because of fiber deficiency, the gut microbiota fed on the colonic mucosa layer that originally acted as a defense barrier against pathogens, leading to heighted pathogen susceptibility. Multiple features of alterations in the gut microbiota composition after a low FODMAP diet are reported, such as a lower total bacteria load, a lower absolute abundance of luminal *Actinobacteria*, *Bifidobacteria*, *Clostridium cluster IV*, *Faecalibacterium prausnitzii*, and a lower concentration of the SCFA butyrate[42-44]. These studies all report on short-term interventions compared to baseline or habitual diets. The inconsistency of the study results are intriguing. McIntosh *et al*[45] comparing a low and a high FODMAP diet found a higher bacteria richness and diversity of Actinobacteria, Firmicutes, and Clostridiales in patients with IBS-D/-M in the low FODMAP group, while a high FODMAP diet decreased the relative abundance of gas-producing bacteria[45]. The sparse documentation of the long-term consequences beyond 8-12 wk of FODMAP restrictions does evoke certain skepticism. The highly restrictive nature of the diet may lead to disordered eating habits and demands much effort and motivation from patients. These factors highlight the importance of reintroduction of FODMAPs after the strict phase in the clinical management of patients with IBS[46]. Inter-individual variability and high inconsistency between clinical findings have made the search for a microbiota signature to predict treatment outcome challenging[47]; and (3) Systemic immune and inflammation responses may also contribute in symptom generation in IBS. McIntosh *et al*[45] showed that urinary histamine levels were substantially reduced following a low FODMAP diet[45], leading to hypothesizing that a low FODMAP diet might be beneficial in a subset of patients with a particular microbiota profile leading to high histamine production, hence where histamine is being a pathophysiology modulator of importance[32,45]. Our group were the first to report reduced levels of pro-inflammatory IL-6 and IL-8, but not TNF-α, after a 3-wk low FODMAP diet. Simultaneously, selected bacteria associated with anti-inflammatory properties, *e.g. Faecalibacterium prausnitzii* and Bifidobacterium, total levels of SCFAs and n-butyric acid, decreased[43]. Others have reported that SCFAs may have anti-inflammatory and immunomodulatory effects, as summarized by Tan *et al*[48], indicating that our findings present a paradox. Indeed, the full connection between diet intake, gut microbiota and its metabolites, and immune and inflammatory responses remains elusive. Herein, the intestinal barrier, gut integrity and low-grade inflammation is further discussed in section "Intestinal barrier and gut integrity", below.

Fecal microbiota transplantation

In fecal microbiota transplantation (FMT) screened stool from a healthy donor is transferred to a recipient with the purpose of altering the diversity of the gut microbiota. FMT is recommended as a therapeutic strategy in *Clostridioides difficile* (CDI) infection, and has also been demonstrated effective in inflammatory bowel disease and IBS[49]. There are multiple routes of FMT delivery available including colonoscopy, nasogastric tube, nasoduodenal tube, enema and oral capsules. Each of these modalities has been associated with varying clinical success. Additionally, whether the donor sample is fresh or frozen, or derived from a related or unrelated donor may result in different outcomes. Many recent randomized controlled trial (RCT) studies in IBS have been published, although with conflicting results. In a meta-analysis of five RCTs, overall FMT did not significantly improve IBS symptoms[50]. Here, the results were largely contradictive; one study showed amelioration of symptoms with FMT over placebo, while another study demonstrated superiority of placebo over FMT. The explanation for such contradictory results may be due to the heterogeneity of the disease. Another explanation may be the route of FMT administration. A recent double blinded RCT recruited 90 IBS patients and randomly assigned them to active treatment (n = 60) or placebo (n = 30) where fresh transplant was delivered with colonoscope to coecum[51]. FMT induced significant symptom relief in

patients with IBS, compared to controls. In 2020, our group investigated the effect of a single FMT using different stool dosages (30 g and 60 g) of frozen feces, delivered to the distal duodenum through a gastroscope. Placebo was the patient's own (autologous) feces. Here, patients responded best to the higher dosage. This study concluded that utilizing a well-defined donor with a normal dysbiosis index and a favorable specific microbial signature is important for a successful FMT[52]. Data on long-term follow-up post FMT in IBS have been sparse. However, 1-year effects were recently reported by Holvoet *et al*[53]. In a doubled blinded RCT of patients with treatment-refractory IBS with predominant bloating, patients were randomly assigned to single dose nasojejunal administration of donor stools or autologous stools[53]. Here, FMT relieved symptoms compared to placebo (autologous transplant), although the effects decreased over 1 year. A second FMT restored the response in patients with a prior response. Evidently, fecal samples from responders had higher microbiota diversity before administration of donor material than fecal samples from non-responders and distinct baseline composition, but unfortunately, no specific marker taxa were associated with response[53]. In addition, 5-year effects were recently reported in a retrospective analysis by Cui *et al*[54]. In this single-center retrospective study, patients with all subtypes of IBS were assigned to receive FMT through nasojejunal administration, colonoscopy administration, or freeze-dried capsules from healthy, screened donors[54]. Considering all patients, regardless of route of administration, 50% of patients reported gradual symptom improvement after one month to 70% and 75% after one and two years, respectively. After five years, 60% of patients experienced improvement. This decline suggests that repetitive FMT may be required for a sustained effect[54].

There are many hopes for the future in IBS treatment, and FMT capsules are one of them. Capsules are beneficial because the route of administration is much less demanding than endoscopy, they put much less stain on the patient, and can be orally administered by the patients themselves at home. Halkjær *et al*[55] performed a RCT study in patients with moderate-to-severe IBS. FMT resulted in altered gut microbiota composition, but patients in the placebo group experienced greater symptom relief compared to the treatment group after three months. Supporting this, Aroniadis *et al*[56] also found that placebo capsules did not induced symptom relief compared with placebo. Hence, the efficacy and safety of FMT in IBS is still under evaluation. Most researchers and clinicians strongly believe that more research is required before the FMT can become an openly available treatment option. A significant question that remains to be answered is whether the described dysbiosis in IBS is a consequence rather than cause of MGB-axis dysfunction in IBS. The varying abovementioned results may indicate that altering gut microbiota is not enough to obtain clinical improvement in IBS. FMT is a highly requested treatment among patients with IBS, and many practitioners find it difficult to refuse patients treatment that may be beneficial. However, researchers are calling for caution on FMT as a treatment of IBS[57]. With reference to safety, patients have reported adverse effects of abdominal pain, cramping or tenderness, diarrhea or constipation, in contrast to 2% in the placebo group[52]. In march 2020, the US Food and Drug Administration issued a warning of potential risk for serious infections due to FMT caused by enteropathogenic or Shigatoxin-producing *E. coli* that occurred following investigational use of an FMT product supplied by a stool bank, from pre-screened donors[58]. Hence, different study designs with larger cohorts are required to examine the efficacy and safety of FMT in IBS.

Indeed, the complex interplay between the host and gut microbiota is not fully elucidated, but certain features in IBS are documented to be involved, including luminal interactions and its pivotal role in the regulation of the immune system. Whether altered microbiota composition, function and abundance is the cause or a consequence of IBS, we need to understand more about their interplay with us as hosts, and importantly, the intestinal barrier and gut integrity in IBS.

INTESTINAL BARRIER AND GUT INTEGRITY

Molecular biology has revealed the presence of structural and functional alterations of the intestinal epithelial barrier and mild activation of the immune system both locally in the intestinal mucosa and systemically, in IBS. We now know that changes in intestinal permeability create a passage for microbiota and their metabolites from the lumen to the ENS, immune cells and systemic circulation, features that are associated with low-grade inflammation in IBS. Intestinal barrier dysfunction is present in a

significant proportion of reported IBS studies, especially in the IBS-D and post-infectious subtype[59]. The association between impaired barrier function and symptoms in IBS are not fully understood but visceral hypersensitivity and pain is possibly explained by exposure of the submucosal neuronal and immune apparatus. Under normal conditions, the intestinal barrier consists of a monolayer of polarised epithelial cells, coated with a thick layer of mucus[60]. As a part of the host defence system, the mucus layer entraps pathogens and is inhabited by commensal microbes such as *Bacteroides*, *Firmicutes,* and *Lactobacillus* whose products, such as IgA, contribute to the prevention of pathogen colonization[61]. Microbiota also produce proteases and protease inhibitors that can modulate the host immune response. In IBS, dysbiosis-derived proteases are thought to contribute to loss of barrier function, immune activation, and symptom generation through activation of protease-activated receptors (PARs)[62]. Recently, higher levels of fecal proteolytic activity has been identified in IBS, particularly in patients suffering from post-infectious IBS[63]. Notably, this was associated with changes in microbiota composition, suggesting that specific microbes contribute to increased production or inadequate suppression of proteases and subsequent activation of PARs that may lead to intestinal barrier dysfunction.

In normal barrier permeability, the space between epithelial cells are sealed by tight junctions and maintained by a complex network of protein interactions. In IBS, the expression levels of tight junction proteins, such as Occludin, Zonula occludens-1, and Claudin-1, have been found to be reduced in the duodenum, jejunum and colon[59,64]. Interestingly, microbiota has been found to regulate the expression of tight junction proteins[65], and enhanced bacterial passage over the barrier has been observed[66].

In the intestinal mucosa, mast cells comprise 2%-3% of the immune cell pool of the lamina propria, and increased intestinal mast cell concentration or activation is one of the most consistent pathological findings in IBS[67-69]. Mast cells possess a great number of stimulatory molecules which allow interaction with a multitude of partners, including immune and non-immune cells. Activated mast cells release mediators such as histamine, serotonin, proteases, and prostaglandins, and they also secrete cytokines and chemokines. Their interactions are indeed complex. Some mast cells interact with both the commensal microbiota and the nervous system by signalling to enteric neurons though serotonin while being influenced by neurotransmitters such as substance P or noradrenalin[70]. In IBS, mast cell-induced activation of enteric neurons may contribute to visceral hypersensitivity[66,71]. Indeed, some bacteria specifically affect mast cells function and activation, but the role of dysbiosis-mast cell-interaction in IBS is yet to be elucidated.

A potential marker of low-grade inflammation in IBS is altered levels of cytokines. Multiple cytokine profiles of patients with IBS have been reported, but they are highly inconsistent. Some has found increased levels of circulating pro-inflammatory cytokines such as IL-6, IL-8, IL-17, and TNF-α[72-74], or reduced levels of the anti-inflammatory cytokine IL-10[72]. Other studies indicate no difference between patients with IBS and healthy controls [75,76]. Interestingly, associations between altered cytokine profiles and changes in gut microbiota have been observed. A study by Hustoft *et al*[43] found reduced levels of IL-6 and IL-8 and decreased levels of Actinobacteria, Bifidobacterium, and *Faecalibacterium prausnitzii* after three weeks of the low FODMAP diet. Changes in cytokine levels could thus indicate an abnormal mucosal immune response associated with changes in the gut microbiota.

Increased expression of Toll-like receptors (TLRs) is an interesting finding in patients with IBS[77,78]. These receptors are found on many different cells, including intestinal epithelial cells and immune cells, and they interact in close relation to neural and immune receptors that are involved in the homeostatic regulation in the gut mucosa[79]. TLRs recognise specific microbial components of both commensal and pathogenic bacteria and play a role in immunologic tolerance to commensals and defend against pathogens[80]. In association with increased levels of TLRs, changes in both cytokine profiles and gut microbiota have been observed in patients with IBS, suggesting that an altered microbiota profile may influence TLR expression and immune activation[78].

Indeed, dysbiosis may induce intestinal barrier loss and increased intestinal permeability that cause bacterial products and metabolites to permeate the epithelial barrier, thus triggering an inflammatory response[81]. Mast cells are ''gate keepers'', and they are not only involved in allergic reactions, but also in host defence including recruitment and activation of other immune cells which may evoke the symptom generation. We believe that further studies should be more focused on which triggering factors that are involved in the link between gut microbiota, intestinal permeability, and intestinal mucosal response in patients with IBS. Indeed, changes in

intestinal permeability create a passage for microbiota and their metabolites from the lumen to the ENS, immune cells, and systemic circulation, features whose effect on the brain should also be investigated.

NEUROIMAGNING AND GUT MICROBIOTA IN IBS

The MGB-axis represent a paradigm shift in both neuroscience, gastroenterology and systems medicine. See Mayer *et al*[82] for a visionary and integrative systems-biology-based model approach to IBS. GI symptoms such as heartburn, indigestion, acid reflux, bloating, pain, constipation, and diarrhea can be triggered by emotional and psychosocial factors. Conversely, GI symptoms alter CNS processing in the absence of detectable organic disease and are implicated in neurological disorders and psychiatric conditions such as anxiety, depression, autism spectrum disorder (ASD), and Parkinson's disease. Brain imaging modalities and techniques are valuable tools that can non-invasively extract both structure and function in the living brain at the millimeter scale and at a temporal resolution down to seconds. To study the IBS brain, or more generally, applying brain imaging to explore disorders of gut-brain interaction and relation to gut microbiota, the most important modality with whole brain coverage is likely magnetic resonance imaging (MRI)[83,84]. Among the plethora of MRI measurement techniques, there are (1) Structural MRI (sMRI), providing 3D images with high spatial resolution and various types of soft tissue contrast enabling quantitative assessment of brain morphometry such as volumes of different brain structures or regions, local and patch-wise cortical thickness and gyrification, and localized MR signal intensity patterns, *e.g.*, radiomics[85]; (2) diffusion MRI (dMRI), measuring directional and tissue-dependent water diffusion at the microscopic scale enabling quantitative assessment of tissue microarchitecture, *e.g.*, metrics such as fractional anisotropy (FA) derived from the voxel-wise diffusion tensor estimation, and large-scale structural connectivity between brain regions obtain by fiber-tracking algorithms; and (3) functional MRI (fMRI), based on blood-oxygen-level-dependent (BOLD) contrast imaging sensitive to local neuronal activity across the brain in situations where the brain is exposed to cognitive, emotional or sensory stimuli given under experimental control, or being in "resting state" where brain activity is assumed to be intrinsic due to spontaneous fluctuations in the paramagnetic BOLD signal and thereby detectable even in the absence of an externally prompted task or a specific sensory stimulus. A large proportion of neuroimaging studies, partly also targeting IBS, have focused on structural and functional brain connectivity using a combination of dMRI and/or fMRI recordings and topological network analysis based on graph theory[86-89], and more recently also deep learning methods and graph convolution approaches for functional annotation of cognitive states[90,91]. It is expected that these deep learning methodologies will also penetrate imaging and network-based analysis in IBS research[82].

Keeping the systems view on IBS, the term *radiomicrobiomics* was coined by De Santis, Moratal and Canals in their perspective paper on advancing along the gut-brain axis through big data analysis for diagnostic and prognostic purposes[92]. The term was introduced with reference to the efforts of combining microbiota sequencing data from the gut microbiota with imaging-based features that can be obtained from the conversion of brain images into mineable tabular data or graph representations in a network context. Interestingly, the gut microbiota seems to influence complex physiological systems other than the gut-brain axis and the pathophysiology of IBS, systems that are homeostatically regulated, partly involving CNS and ANS processing. These are blood pressure and the development and pathogenesis of hypertension, glycemic control, development of obesity and diabetes, mood regulation, and anxiety and depression[93-96]. In all these cases, neuroimaging and network analysis will be an important window to the brain and its interplay with microbiota composition and dynamics. More specifically, exploring the associations between neuroimaging parameters, such as brain regional volumes and gray matter densities assessed with sMRI, microstructural patterns assessed with dMRI and derived FA-values, or interregional functional connectivity in *e.g.* the salience network, visceroceptive, pain processing, or emotion-regulating networks assessed with resting state fMRI and specific gut microbiota signatures, has the potential of vastly enhancing our knowledge on gut-brain interactions in IBS. In neuroimaging of the IBS brain, one of the most consistent findings are alterations in the structure and function of key regions of the somatosensory network, including the globus pallidus, putamen, and caudate, composing the basal ganglia[83]. It has also been reported increased gray matter

density (GMD) in the hypothalamus and decreased GMD in the prefrontal cortex in the IBS brain[97]. In rectal distention experiments, patients with IBS had a differential brain response in the pain matrix and default mode network[89]. Neuroimaging studies has also revealed gender-differences in IBS brain network alterations. Female IBS patients showed increased cortical thickness in the pre- and post-central gyrus and decreased thickness in the bilateral insula and the left subgenual anterior cingulate cortex (sgACC), compared to healthy female controls. Connecting emotions and altered brain function in IBS, patients with IBS and comorbid alexithymia have different brain responses to rectal distention in the right insula[98]. As a first in our field, Norlin *et al*[99] recently provided evidence that the vulnerability to fatigue in IBS is associated with connectivity within a mesocorticolimbic network as well as immune activation in the form of enhanced plasma levels of TNF-α, compared to controls. Indeed, there has been published a large series of papers on brain imaging in IBS, and now, evidence for disrupted subcortical and cortical regions mediated by gut microbial modulation are emerging. Labus *et al*[100] reporting associations between brain region-to-region functional connectivity and microbiota found a correlation between Clostridia and Bacteroidia with connectivity of the thalamus, the basal ganglia (caudate nucleus, putamen, pallidum, nucleus accumbens), the superior part of the precentral gyrus, the anterior insula and ventral prefrontal regions. Recently, the same lab also reported on fecal metabolites and resting state fMRI[101]. Here, the differences in histidine, cysteine, glycine, glutamate, spermidine, and anserine were significantly associated with the alteration in left dorsal part of the posterior cingulate gyrus to the left putamen. Also, the changes in histidine, tryptophan, uracil, 2-deoxyuridine, thymidine, and succinate were differentially associated with the alteration in the right superior frontal gyrus to the right putamen. Interestingly, this interaction may be mediated by aberrant tryptophan signaling in IBS, which is important because it is a substrate for serotonin synthesis.

In combining brain imaging data, molecular and genetic data, and metagenomic data for joint analysis, new challenges and opportunities arise in the attempt to elucidate the mechanisms and biomarkers of IBS. This endeavour is further described and discussed in the section "Big data analysis" below.

INTESTINAL MICROBIOTA ANALYSIS

Importance of method selection

Microbiome research is advancing rapidly, improving the precision of taxonomy and functional surveys and minimizing methodological limitations. After almost two decades of the earliest intestinal microbiota surveys in humans, we have advanced towards recommendation of quasi-standard methodological procedures to make next-generation sequencing (NGS) data comparable across studies. Notwithstanding, the complete implementation of standards is challenging, given the wide variety of commercial options for sampling, DNA extraction, amplicon generation, library preparation, and DNA sequencing that users fit at their own convenience, making cost-effectivity prevailing. In this regard, the International Human Microbiome Standards consortium has agreed that stool sampling requires minimal processing (no addition of preservation buffers and nuclease inhibitors) to maintain microbiota DNA and RNA integrity, thus facilitating sampling, storage and transport logistics by donors and patients. Besides, stool sub-sampling was revealed to produce minimal variation within individuals[102,103]. Among the multiple methodological steps, DNA extraction introduces the larger variation across experiments, and chemical (muralytic enzymes) or physical cell-wall disruption (bead-beating) methods during DNA extraction are recommended to gain the representation of some microbial species during massive and parallel sequencing and taxonomy data appraisals[103,104]. Taxonomy surveys *via* amplification and sequencing of bacterial 16S rRNA gene are widespread because of their cost-effectivity. A large collection of tools (*e.g.*, QIIME2, Mothur, DADA2, *etc.*) and reference repositories (SILVA, RDP, Greengenes, GTDB) have been developed for such an aim. However, this methodology allows reliable identifications mostly at the family and genus levels. Also taxonomy classification depends on the region amplified[105,106] and, accordingly, inconsistencies have been found across studies. Currently, most of the studies are sequencing the V4 or V3-V4 hypervariable regions because of their larger genetic variation and discriminatory power, facilitating re-use and comparisons across different studies.

Metagenomic analysis, based on non-targeted massive DNA sequencing, outperforms the 16S rRNA gene hypervariable region sequencing, although it is much more expensive. Gigabase-level information normally recovered from individual samples permit inspection of microbial species present in human samples and other ecologically complex environments[107,108]. The discriminatory capability of this approach is constantly improved thanks to the existence of comprehensive genome catalogues[109], compiling a huge amount of microbial genetic variability and making it possible sample profiling at the strain level[110]. In addition to the detailed taxonomy surveys, metagenomics makes functional appraisals feasible *via* DNA read mapping strategies using curated and comprehensive repositories (*e.g.*, KEGG, COG, eggnog databases). The functional analysis has also been developed for 16S rRNA gene amplicon sequencing data (namely PICRUSt). However, the predictions made with such an approach have a high degree of uncertainty due to the ambiguous taxonomic assignments of the 16S rRNA readouts and absence of functional variation information at the species level (minimal gene/functions shared on multiple genome examinations – pangenome – within a single species).

Metadata does matter

The interpretation of microbiome data require a proper control of covariates that many times are not available to be incorporated into the data analysis. This could lead to ambiguous (relying on the generally recognized strain-specific pathogenicity traits) and uncertain (plenty of false-positives) associations between the microbiota and health and disease states, largely influenced by confounding variables[111-113]. Of environmental factors, the intestinal microbiota is strongly influenced by the dietary patterns. Therefore, the use of dietary records around the sampling time are good strategies to integrate such information in the data analysis. The value of this type of information is even more important in microbiota-based biomarker discovery for example for IBS given the impact of food intake on disease symptoms[114]. Nevertheless, not all studies have found meaningful differences in microbiota when using dietary records[115,116]. There is growing evidence supporting a role of energy and macronutrient intake on the intestinal microbiota which could affect associations with health and disease. Body mass index (BMI), gender and age could affect both dietary habits and the intestinal microbiota and change through life differently in women and men[117-119]. By integrating gender, BMI, diet, and age information with microbiota data, the results are less influenced by the subjects' idiosyncratic variation and signals looking for links between gut microbes and health/disease states become more reliable. Moreover, pharmacological treatments given to IBS patients to tackle symptoms should be considered as they could bias the conclusions on the microbiota signatures correlated and playing a role in IBS. There is indeed extensive impact of non-antibiotic drugs on the composition and metabolic function of the gut microbiota [14,120]. In summary, good practices in microbiome research for clinical application undoubtedly involve a meticulous metadata recording covering a large set of individual and lifestyle information that permit uncover unquestionably the influence of gut microbes in our health.

Perspectives for improving microbiome role predictions

The integration of functional omics provides information on the potential role of intestinal microbes and the metabolic products resulting from host-diet-microbe interactions and allows generating human-data-driven hypotheses which could be latter validated in study models. These methods turn data processing more complex because of multidimensionality, but provide clues on the molecular mechanism driven by microbe-host interactions and underlying health and disease[121]. For instance, the correlations of gut microbes (metagenomics) with secondary bile acids (lipidomics), SCFAs (metabolomics), and pro-inflammatory molecules (proteomics/cytokine arrays) make it possible to distinguish microbial groups that plausibly explain disease states or the physiological response to a particular dietary components[122-124]. The high individual specificity and variability of the microbiome data also requires the application of statistical methods that minimize false-positives during biomarker discovery, permit an adequate covariate control and integrate other multidimensional datasets. In this context, the EU COST action ML4Microbiome represents an interesting initiative for advising, researching, and developing advanced statistical and machine learning approaches applied to microbiome research that could greatly contribute to standardizing and improving data analysis in this field[125,126].

Promising developments have also emerged to improve, for example, the accuracy of the dietary assessments. These are often based on self-reported data and, therefore, biased affecting the interpretation of its relationship with the microbiome and health

outcomes. In this regard, metabarcoding of plant DNA has been proposed as a method to tacking human plant intake more accurately than using dietary questionnaires. Although this strategy has been only applied to gain information on plant components of the human diet, it looks promising to infer the dietary intakes and the resulting diet-microbe interactions[127].

Sequencing technological advances are also helping to improve the taxonomy resolution of targeted amplicon-based microbiota analysis. The emergence of single-molecule sequencing platforms (Oxford Nanopore Technologies and PacBio) has permitted to generate longer DNA reads, pivotal to increase the information of gene markers under inspection in microbial diversity assessments and to gain resolution at the species-level at a lower cost than metagenomics[128,129]. This methodology has already demonstrated good performance on microbiota surveys despite the modest per-base quality of its reads compared to the classical Sequencing-by-Synthesis (SBS)-based instruments (Illumina). Its potential is even more promising to infer strain-level variation pivotal to determine, for example, species engraftment after FMT[130], which, as mentioned above, is under investigation to treat IBS[52,131].

BIG DATA ANALYSIS

The enormous potential of big data, when harnessed efficiently by powerful statistical methods, mathematical models, and machine learning algorithms, often translates into deeper insights in multi-factorial dynamic systems, which are otherwise complicated to explore, describe and comprehend. The MGB-axis is a well-suited example of such a complex multivariate system and data science *(i.e.*, machine learning algorithms and statistical analyses) is the method of choice to approach this problem. It has become highly evident that no single factor underlie the heterogeneous disorder of IBS. Its investigation requires analysis of large datasets collected from an array of clinical disciplines for a deeper understanding of pathophysiological mechanisms and pathways, and correlations with specific symptoms and symptom severity.

The large number of factors involved both from the brain and microbiota along with their continuous variability, yield large amount of information. These factors are probed using various clinical modalities across several points in time in longitudinal research. The number of resulting variables can range from few hundreds to tens of thousands and the magnitude of data can easily approach hundreds of thousands to several millions of data points, even for studies recruiting several tens of participants. This sheer scale of data combined with larger dimensionality and significant variability sets up a ripe case for employing sophisticated data science methods to study intricate relationships between brain and gut microbiota.

The MGB scientific community appear to already recognize and acknowledge the importance of data science in the field of our research. For example, in their review, De Santis *et al*[92] described the potential of large amount of information emerging from advanced neuroimaging systems and sophisticated microbiome sequencing techniques to probe complex interactions in the MGB-axis. Their proposal was to combine data from both of these domains to analyze quantitative features for intricate relationships using computational analysis methods, a process they termed “radiomicrobiomics”, which could potentially unveil novel biological information on the MGB-axis. Similar ideas were expressed in another critical review whose key note suggested that encoding microbial information along with other necessary variables into machine learning algorithms could excel our understanding on GI disorders, which are challenging to diagnose due to multifactorial nature of underlying pathology[132]. Mayer *et al*[83,133] had stressed the need of integrating large sets of host's multi-omics data and microbial data with machine learning techniques to reveal novel insights into the MGB-axis, independent of existing theories and hypothesis. Kaur *et al*[134] also emphasized the role of machine learning in multiomics data analysis to probe MGB relationship and discussed a framework to move beyond prediction to prevention and personalized therapeutics in MGB related disorders.

In line with these proposals, reports of initiatives and on-going work where the MGB-axis is being explored using the discipline of data science are beginning to emerge. In a recent study, statistical analyses were performed on combined brain and microbial datasets, acquired using resting state fMRI and genetic sequencing, respectively[135]. Probably for the first time, clear correlation-based associations were drawn between certain species of microbiota and corresponding brain regions affected by it, a step forward in the right direction. Wu *et al*[136] studied the association of gut microbiome in ASD using an array of statistical and machine learning-based analyses

and realized presence of certain bacterial genera in ASD group, which could be used as a potential ASD biomarker. Stevens *et al*[137] studied association of depression phenotype with gut microbiome using microbial genetic information at single nucleotide resolution using multivariate analysis. Based on genetic data of microbiome, they were able to differentiate between depression and healthy cases. The Bergen brain-gut-microbiota study is a notable example of an on-going work that integrates data science with multiomics, where both brain and gut data is being collected from an IBS patient cohort and healthy controls, as shown in Figure 1[138]. BrainGutAnalytics, an advanced analysis project under the umbrella of the Bergen brain gut study, aims to apply sophisticated data science methods to locate IBS biomarkers in brain and peripheral organ systems[139].

Despite these rapidly growing applications of data science in investigating the MGB-axis, it appears that the full potential of data science is yet to be leveraged. Data science techniques, such as machine learning models, particularly thrive in scenarios where the sample size is high (*i.e.*, in order of thousands or more), as it allows the models to adequately learn the underlying data structure by iterating over large number of observations. On the contrary, clinical studies are often limited by sample size albeit high dimensionality of data, as various studies report participant cohorts comprising a few tens to a few hundred subjects only[120-122]. This limited sample size, on one hand, impedes the development of reliable computer models and on the other, high feature to sample ratio could lead to overfitting of the model, which often result in misleading predictions[119]. One tangible way to address this problem is aggregation of several datasets coming from various small scale studies into a larger MGB-axis database, as also proposed by other researchers[80,118,121]. Such a collection will not only feed the needs of data starving computer models but will also represent diverse sectors of subject population in terms of demographics and genetic backgrounds, improving generalization and validation of analysis outcomes. However, such an initiative would only be meaningful if a highly controlled and uniform system of data collection could be developed and implemented across all participating studies. A merger of various datasets taken from isolated studies following their own highly customized protocols, based on variable inclusion and exclusion criteria, will not carry much scientific value. Similarly, an acceptable level of consistency in data management system across all studies is imperative for rapid data accessibility, interpretation and interoperability. The establishment of a larger MGB database would also facilitate much needed interdisciplinary collaboration in MGB research, as data scientists and clinicians must join forces together to solve this complex puzzle.

CONCLUSION

In recent years, research aiming to understand the influence of the gut microbiota on bidirectional interactions between the gut and brain has gained momentum. As described and discussed in this review, the role of microbiota in IBS is so multifaceted that it requires research approaches across disciplines and scientific fields, to reveal details of the complex interactions. Currently, most dietary or FMT interventions are limited to observations of transient microbial shifts within short time frames. Personalized responses of the host microbiota may explain some of the heterogeneity of research outcomes, but not all. Because IBS fluctuates between periods of remission and aggravation of symptoms, longitudinal sampling, multiple sample time-points, post-intervention follow-ups and washout periods for cross-over studies are needed to identify microbial changes that are missed when using cross-sectional sampling, will be of great importance in future studies. We know that gut microbiota profiles are significantly associated with alterations in intestinal gut integrity, brain microstructure, intrinsic neural activities, and cognitive function and mood. How this tremendously intricate symbiotic relationship works in IBS, remains to be unraveled. Multimodal and interdisciplinary clinical studies that include assessments of the gut microbiota composition and function in conjunction with neuroimaging and behavioral testing, such as the Bergen BrainGut microbiota-study[138] are necessary for verification of directionality and causality in the MGB-axis in IBS. Other important work to come are how probiotics influence gut microbiota and affect functional changes in the brain through gut microbiota[140]. As described in this review, the choice of method for analysis is important. We believe, that only through integration of multiple advanced techniques, such as metabolomics and neuroimaging, can we generate a complete picture of host and microbiota pathways in IBS.

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REVIEW

Sarcopenia in hepatocellular carcinoma: Current knowledge and future directions

Abhilash Perisetti, Hemant Goyal, Rachana Yendala, Saurabh Chandan, Benjamin Tharian, Ragesh Babu Thandassery

ORCID number: Abhilash Perisetti 0000-0003-4074-6395; Hemant Goyal 0000-0002-9433-9042; Rachana Yendala 0000-0002-5615-5525; Saurabh Chandan 0000-0002-2661-6693; Benjamin Tharian 0000-0002-7797-3738; Ragesh Babu Thandassery 0000-0002-5357-4847.

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Abhilash Perisetti, Benjamin Tharian, Department of Internal Medicine, Gastroenterology and Hepatology Division, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

Abhilash Perisetti, Department of Interventional Oncology and Surgical Endoscopy, Parkview Health, Fort Wayne, IN 46825, United States

Hemant Goyal, Department of Internal Medicine, The Wright Center for Graduate Medical Education, The Wright Center for Graduate Medical Education, Scranton, PA 18501, United States

Rachana Yendala, Department of Hematology and Oncology, Conway Regional Medical Center, Conway, AR 72034, United States

Saurabh Chandan, Department of Internal Medicine, Gastroenterology and Hepatology Division, CHI Creighton University Medical Center, Omaha, NE 68107, United States

Ragesh Babu Thandassery, Department of Medicine, Central Arkansas Veterans Healthcare System, Little Rock, AR 72205, United States

Corresponding author: Abhilash Perisetti, FACP, MBBS, MD, Academic Fellow, Department of Internal Medicine, Gastroenterology and Hepatology Division, University of Arkansas for Medical Sciences, 4301 W Markham St., Little Rock, AR 72205, United States.
abhilash.perisetti@gmail.com

Abstract

Liver cancer is the second most occurring cancer worldwide and is one of the leading causes of cancer-related deaths. Hepatocellular carcinoma (HCC) is the most common (80%-90%) type among malignant liver cancers. Sarcopenia occurs very early in HCC and can predict and provide an opportunity to improve muscle health before engaging in the treatment options such as loco-regional, systemic, and transplant management. Multiple prognostic stating systems have been developed in HCC, such as Barcelona Clinic Liver Cancer, Child-Pugh score and Albumin-Bilirubin grade. However, the evaluation of patients' performance status is a major limitation of these scoring systems. In this review, we aim to summarize the current knowledge and recent advances about the role of sarcopenia in cirrhosis in general, while focusing specifically on HCC. Additionally, the role of sarcopenia in predicting clinical outcomes and prognostication in HCC patients

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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undergoing loco-regional therapies, liver resection, liver transplantation and systematic therapy has been discussed. A literature review was performed using databases PubMed/MEDLINE, EMBASE, Cochrane, Web of Science, and CINAHL on April 1, 2021, to identify published reports on sarcopenia in HCC. Sarcopenia can independently predict HCC-related mortality especially in patients undergoing treatments such as loco-regional, surgical liver transplantation and systemic therapies. Basic research is focused on evaluating a balance of anabolic and catabolic pathways responsible for muscle health. Early clinical studies have shown promising results in methods to improve sarcopenia in HCC which can potentially increase prognosis in these patients. As sarcopenia occurs very early in HCC, it can predict and provide an opportunity to improve muscle health before engaging in the treatment options such as loco-regional, systemic, and transplant management. Further, sarcopenia measurement can obviate the confounding caused by the abdominal ascites in these patients. The use of sarcopenia can add to the existing scoring systems to better prognosticate the HCC.

Key Words: Sarcopenia; Skeletal muscle; Hepatocellular carcinoma; Cirrhosis; Outcomes; Liver cancer

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Core Tip: Sarcopenia is a condition defined by the loss of skeletal muscle mass, quality and strength. It is commonly seen as a part of normal aging but can also be noted in multiple conditions such as chronic inflammation, cancers and use of drugs. Sarcopenia is common in liver cirrhosis and is associated with overall poor outcomes (disease-free survival). Recently, the adverse effects of sarcopenia in hepatocellular carcinoma (HCC) has been an area of intense interest. Altered bio-impedence and rapid muscle loss in liver diseases could alter skeletal muscle strength in these patients. Additionally, development of tumor-related cytokines can accelerate the sarcopenia progression which could provide insights into disease progression and response to various therapeutic options. While multiple scoring systems are available to evaluate the HCC progression, sarcopenia provides an additional functional status tool to further refine these systems. In this article, we summarize the role of sarcopenia in HCC progression and changes during locoregional and systemic treatments.

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INTRODUCTION

Primary liver cancers include hepatocellular carcinoma (HCC) and other non-HCC tumors. Primary liver cancers are the second most lethal cancer worldwide, fourth leading cause of cancer mortality and sixth frequently diagnosed cancer per year[1]. HCC is the most common cancers among the primary liver cancers, which constitutes 90% of cases. HCC usually develops within a liver cirrhosis (cirrhotic-HCC, 80% of cases), and rarely with no appreciable cirrhosis or advance fibrosis (non-cirrhotic-HCC, 20% of cases)[2]. Due to aggressive nature of HCC, prognosis is poor. This is compounded by delay in the treatment, limiting life expectancy and management options. Early identification of high-risk features for appropriate stratification, and prognostication in HCC is paramount to alter the disease course and improve survival. Several prognostic staging systems and biomarkers have been developed to identify the patients at risk of poor prognosis[3]. Some of these include Cancer of the Liver Italian Program, Barcelona Clinic Liver Cancer (BCLC), Child-Pugh score, Chinese University Prognostic Index score, the Hong-Kong Liver Cancer stating system and

Japan Integrated Staging. Further, biomarkers such as alpha-fetoprotein (AFP), des-γ-carboxyprothrombin AFP-L3, vascular endothelial growth factor, and angiopoietin 2 were used as independent prognostic factors in advanced tumors[4]. However, current available staging and prognosticative systems lack parameters that consider nutritional, functional and performance status[5]. Although long-term prognosis is dependent on the liver reserve and staging of the cancer, poor performance can significantly affect clinical outcomes in HCC patients. The use of the Eastern Cooperative Oncology Group classification with BCLC could provide an assessment of patients functional status.

Rosenberg[6] introduced the term "Sarcopenia," which was coined from the Greek word "sarx," or "flesh," and "penia," or "loss." It can be defined as loss of skeletal muscle mass, quality, strength with a reduction in the motor unit number, atrophy of type muscle fibers[7], and can contribute to frailty, functional impairment, and disability[8-11]. Three most commonly used diagnostic criteria used for sarcopenia include "muscle mass" (height-adjusted), "muscle strength," and/or "physical performance"[12]. A focus on muscle function has shown to be a powerful predictor of clinically relevant outcomes rather than muscle mass alone[13]. Recently, body mass index (BMI)-adjusted mass is found to be a better predictor of adverse outcomes than height-adjusted muscle mass[14,15]. Further, multiple muscles or groups of muscles could be utilized to assess sarcopenia. Some of the most commonly used muscles include the paraspinal muscle area (psoas muscle, quadratus lumborum, transverse spinal muscle, erector spinae muscles) and triceps muscles (mid-arm circumference). Loss of skeletal muscle mass can affect static, dynamic and isokinetic strength[16]. It can also be associated with a decline in the maximum oxygen consumption (at a rate of 3%-8% per decade of life starting from 30 years) which ultimately leads to a decrease in overall functioning[17]. Dynamic changes in skeletal muscle mass and function can occur with changes in hormones (daily insulin, glucagon), anabolic steroids, corticosteroids, thyroid (month-to-month), and immune mediators [interleukin (IL)-1, tumor necrosis factor, and IL-2]. Primary sarcopenia is noted to be due to physiological states such as aging and secondary causes (acute or chronic illness)[18]. Individuals with cancer may deplete up to 80% of their muscle mass. Further, sarcopenia can be noted in as high as 80% and 60% of patients with upper gastrointestinal and lung cancers, respectively[19]. Pre-therapeutic sarcopenia is noted with highest prevalence in esophageal and small-cell lung cancers and could have severe consequences in terms of post-operative complications, chemotherapy-related toxicity, and poor overall survival (OS)[20].

Cross-sectional imaging is commonly performed in HCC patients for diagnosis, surveillance, and treatment response[19]. It is logical to use this cross-sectional imaging to evaluate skeletal muscle mass simultaneously for valuable information to assess the prognosis and treatment outcomes. Additionally, patients with cirrhosis and HCC commonly develop ascites spuriously increasing the abdominal girth and weight. Despite this increase, significant proportion of these patients have decreased muscle mass leading to "sarcopenic obesity[21]." Use of an objective tool (which is measurable and reproducible) to assess the survival of HCC patients with ascites remains a challenge. Furthermore, methods to assess the prognosis of HCC patients during/after loco-regional (radiofrequency ablation, radioembolization, chemoembolization), liver transplantation, and systemic therapy (chemotherapy, immunotherapy) could have a long-lasting impact on these individuals. One such objective method is to use sarcopenia to assess the patient response and overall could assist in OS in HCC patients[14,22-34]. Therefore, this manuscript aims to describe the role of sarcopenia in the management and prognosis in HCC. Furthermore, we aim to describe and summarize the methods to improve sarcopenia to enhance the survival of patients undergoing treatment for HCC.

LITERATURE SEARCH

An electronic search was performed using databases PubMed/MEDLINE, EMBASE, Cochrane, Web of Science, and CINAHL on April 1, 2021, to identify published reports on sarcopenia in HCC. We used the following search terms- "carcinoma, hepatocellular" or "cancer, hepatocellular" and "sarcopenia" or "sarcopenias". A total of 4762 articles were published on sarcopenia and 167571 on hepatocellular cancers. Both basic science and clinical studies were included. A combined search revealed 2289 articles over the last 12 mo. The authors AP and HG reviewed the articles independently. Clinical reviews, case reports, and case series were excluded. A manual search was

performed by evaluating the references from included studies and related articles in multiple databases. If any discrepancies, these articles were re-reviewed by the author RT. After removing non-relevant/duplicates/non-English language articles, including a manual search, 80 full length published articles were finally reviewed.

HCC AND SARCOPENIA

Secondary sarcopenia is a common finding in patients with cirrhosis. Reduction in protein synthesis can lead to a decrease in lean body mass seen in cirrhotics[26]. Protein catabolism seen in disease processes such as neoplasms can lead to significant loss of muscle mass and it can be seen up to 40% of patients with liver cirrhosis[35]. Sarcopenia can be associated with an increased risk of encephalopathy, post-transplant mortality, infections, treatment effectiveness, and quality of life[36-38]. Patients with cirrhosis who were diagnosis with HCC showed accelerated sarcopenia up to 30-40% at the time of diagnosis[39,40]. Sarcopenia in these patients can independently predict HCC-related mortality along with decompensated cirrhosis, performance status, TNM staging, and BCLC class[41,42]. However, each of these have limitations with biggest being lack of prognostication, inability to provide comprehensive tool to assess complex interactions between cirrhosis, HCC and functional capacity[43]. Further, factors responsible for survival differ significantly among patients with compensated and decompensated cirrhosis[44].

As HCC occurs over time in patients with underlying chronic liver disease, assessment of skeletal muscle mass and change overtime can provide important details about deterioration of the disease. A number of tumor-related factors (cytokines and myokines) can change the skeletal muscle mass which can assist to further refine these scoring systems. Furthermore, cirrhotic have ascites, disproportionate loss of muscle compared to fat (altering BMI) leading to difficulty in interpreting bioimpedance, anthropometric measurements. Hence use of tools to integrate degree of sarcopenia-related measurements by CT-based techniques can offer ways to predict change in these patients[45,46].

BIOLOGICAL BASIS OF SARCOPENIA IN HCC

Sarcopenia is the condition characterized by loss of muscle strength, mass, and functional ability. The pathophysiology of this muscle loss can be multifactorial (hormonal, inflammatory, age-related, chronic liver and non-liver states, drug induced). Loss of muscle anabolic activity with nutritional deficiency can further worsen sarcopenia. Loss of skeletal muscle homeostasis especially between hypertrophy and regeneration can lead to sarcopenia. Most of the changes related to sarcopenia originates with normal aging process. A balance of muscle protein anabolic and catabolic pathways are responsible for muscle health. During sarcopenia, multiple cellular changes occur such as the reduction in myofibres (size and number), myosteatosis (development of intramuscular and intermuscular fat infiltration)[47], decreased number of type II fibre satellite cells. Further, loss of mitochondrial integrity, molecular signaling [IGF-1, mammalian target of rapamycin complex 1 (mTOR)], neurological (plaque formation, motor neuron loss), epigenetic change (modulated *via* microRNAs), endocrine factors (myostatin , osteocalcin and abnormal communication among them) and reactive oxygen species (ROS) imbalance[48] combined with reduced physical activity can all contribute to the muscle loss. Some of the frequent causes of sarcopenia are elucidated in Figure 1. Hyperammonemia, increased autophagy, decreased protein synthesis, abnormal mitochondrial activity, increased proteasomal activity, and low testosterone levels are also responsible for sarcopenia cirrhosis[49,50]. It is compounded by decreased metabolic substrates (especially branched-chain amino acids)[51], extrahepatic gluconeogenesis, and increased insulin resistance/pro-inflammatory cytokines (NFκB signaling, mTOR inhibition, enhanced apoptosis, eukaryotic initiation factor-2[52]. Portal hypertension-related complications and alcohol intake further worsen sarcopenia in cirrhosis[53,54].

Early sarcopenia can be seen in HCC individuals[24,55]. Myokines (myostatin, IL-6, follistatin) are cytokines produced and secreted by muscle fibers and can exert paracrine/autocrine effects[33]. Myokines can exert immunological and anti-inflammatory effects and facilitate proinflammatory state of liver fibrosis, cirrhosis, and HCC development. Although myostatin levels in HCC have been a matter of debate, high IL-6 and follistatin levels had a significantly lower 5-year OS rate in HCC and were

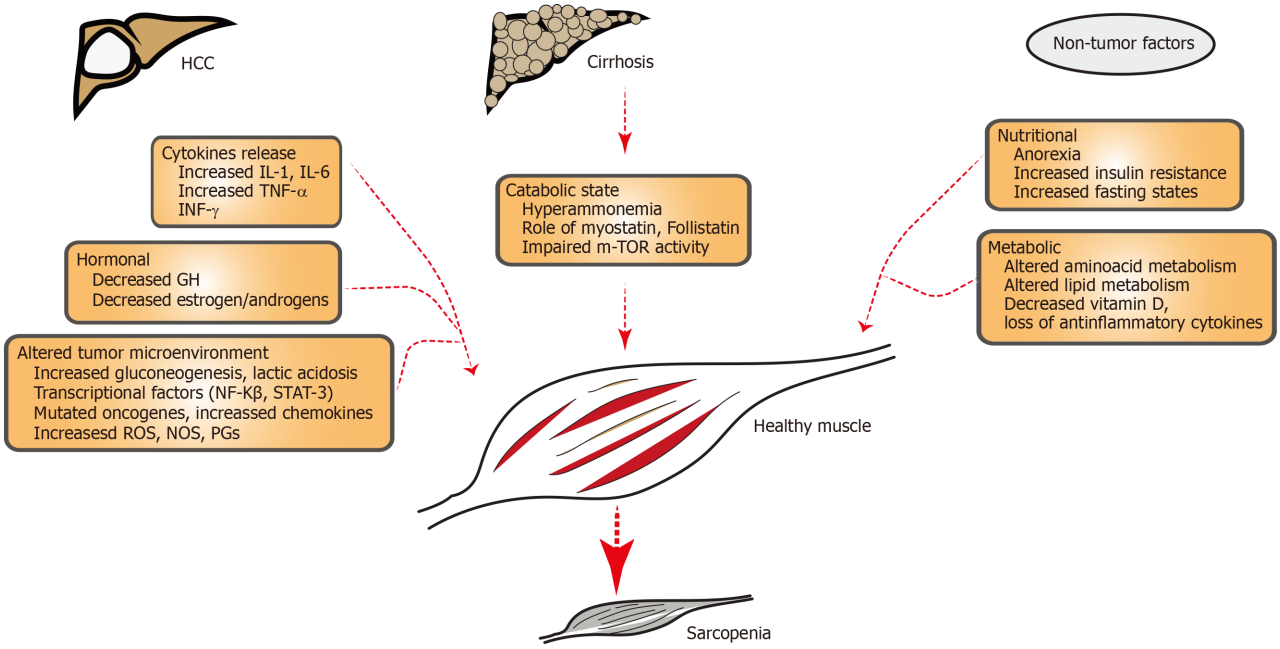


Figure 1 Schematic illustration showing factors contributing to sarcopenia in hepatocellular carcinoma and cirrhosis. Patients with hepatocellular carcinoma have increased release of cytokines, hormonal substances (GH, anabolic steroids) and altered tumor microenvironment (with hypercatabolic state, mutagenesis included by altered DNA, increased reactive oxygen species. Patients with HCC have underlying cirrhosis with hyperammonemia, decreased m-TOR activity which can contribute to sarcopenia. Non-tumor factors include poor nutrition and altered amino acid or lipid metabolism. HCC: Hepatocellular carcinoma; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor alfa; INF-γ: Interferon gamma; GH: Growth hormone; NF: Nuclear factor kappa B; STAT-3; Signal transducer and activator of transcription 3; ROS: Reactive oxygen species; NOS: Nitric oxide species; PGs: Prostaglandins; mTOR: Mechanistic target of rapamycin.

related to tumor progression by BCLC/TNM staging in HCC[33]. Follistatin is a glycoprotein and inhibitor of the TGF-β superfamily (such as myostatin, activin), and it can be related to tumor stage, size and can play an oncogenic role in hepatocarcinogenesis. These details provide important insights into potential agents such as myostatin inhibitors, mitochondrial protective agents, and antioxidants, which can be utilized for liver cirrhosis or HCC[55]. Such anti-sarcopenic treatments could be used to prolong or further reverse molecular, and metabolic changes noted in HCC patients.

CHANGES IN SARCOPENIA WITH HCC TREATMENTS

Sarcopenia in HCC patients undergoing various treatments (locoregional and systemic) has been shown to impact outcomes and survival. Multiple studies have reported outcomes among these patients. It has been showed that a baseline sarcopenia is associated with lack of response to HCC treatments, further decompensation episodes, and increased mortality[56]. In the following sections, we elaborate on studies evaluating the role of sarcopenia in HCC patients with various treatments such as loco-regional, surgery, transplant, and chemo/immunotherapy.

LOCO-REGIONAL THERAPY

Patients with HCC can be candidates for multiple loco-regional treatment (LRT) options such as radiotherapy, chemoembolization, radioembolization. Data on sarcopenic predicting response to LRT is sparse (Table 1)[14]. Iritani *et al*[15] reported 217 HCC patients on LRT and evaluated the role of sarcopenia. In this study, L3 skeletal muscle index (SMI) was used to define sarcopenia. Patients with low L3 SMI showed a significantly lower OS compared to those without sarcopenia (P = 0.004). Further, obese sarcopenic patients died earlier (P = 0.013)[15]. In 2015, Fujiwara *et al*[57] showed a higher risk of HCC recurrence in sarcopenic patients in 515 patients with BCLC stage 0/A who underwent percutaneous radiofrequency ablation (RFA). In 2017, a retrospective study of 182 patients with HCC undergoing percutaneous RFA therapy with curative intent was analyzed[58]. Patient with sarcopenia decreased pretreatment psoas muscle index (PMI) survival (overall cumulative survival) was

Table 1 Outcomes of hepatocellular carcinoma patients undergoing loco-regional therapy with sarcopenia

Ref.	Technique	*n*	Methods and outcomes
RFA			
Iritani *et al*[15] (2012-2014, Japan)	RFA	217	L3-SMI. B36.0 cm^2/m^2 for men and B29.0 cm^2/m^2 for women. Sarcopenia patients had lower OS than those without
Fujiwara *et al*[57] (2015, Japan)	RFA	515	L3-SMI used. B36.2 cm^2/m^2 for men and B29.6 cm^2/m^2 for women. Sarcopenia was associated with a higher risk of recurrence in very early/early-stage HCC who underwent treatment with RFA.
Yuri *et al*[58] (2017, Japan)	RFA	182	PMI used. 6.36 cm^2/m^2 for men and 3.92 cm^2/m^2 for women. Sarcopenia was associated with overall reduced HCC survival with no effect on recurrence.
TACE			
Dodson *et al*[38] (2013, United States)	TACE drug eluding TACE	216	TPA was used to assess sarcopenia. TPA of < 477 mm/m^2 for men and < 338 mm/m^2 for woman. Sarcopenia was independently associated with increased risk of death (lowest *vs* highest TPA quartile, HR = 1.84; $P = 0.04$)
Kobayashi *et al*[60] (2018, Japan)	TACE	102	L3-SMI used. 42 cm^2/m^2 for men and 38 cm^2/m^2 for women. Change in L3-SMI was an independent prognostic factor in patients with HCC treated with TACE.
Loosen *et al*[61] (2019, Germany)	TACE	56	Mean PMI was 11.81 mm/m^2. Low PMI (13.39 mm/m^2) had significantly lower median overall survival (491 d) compared to high PMI (1291 d)
Fujita *et al*[59] (2019, Japan)	TACE	179	PMI used. < 6.0 cm^2/m^2 for men and < 3.4 cm^2/m^2 for women. No difference was normal with low PMI and normal PMI for HCC outcomes. However, changes in PMI were significant after TACE with significant loss of liver function reserves post treatment.
TARE			
Faron *et al*[32] (2020, Europe)	TARE	58	MRI derived FFMA were used to predict sarcopenia. FFMA < 3582 mm^2 for men and < 2301 mm^2 for men. Low FFMA was associated with significantly reduced OS (197 *vs* 294, $P = 0.02$).

Studies depicting various loco-regional treatments utilized in hepatocellular carcinoma in relation to sarcopenia. RFA: Radiofrequency Ablation; TACE: Transarterial chemoembolization; TARE: Transarterial radiofrequency embolization; L3-SMI: Third lumbar vertebrae-skeletal muscle index; OS: Overall survival; HCC: Hepatocellular carcinoma; TPA: Total psoas area; FFMA: Fat-free muscle area; PMI: Psoas muscle index.

51.5% compared to 86.5% without sarcopenia ($P < 0.0001$. In addition to sarcopenia, total bilirubin ≥ 1.2 mg/dL, des-γ-carboxy prothrombin ≥ 34 mAU/mL ($P = 0.009$) were found to be adverse predictors of OS [58]. These findings were irrespective of CTP score or achievement of SVR in HCV-related HCC. Furthermore, above findings indicate the usefulness of sarcopenia to assess outcomes of HCC patients undergoing RFA.

Trans-arterial interventions for the HCC can be chemoembolization (TACE) or radioembolization (TARE) and are increasingly being utilized for large or multifocal disease with metastasis or macrovascular invasion[27]. The available data are conflicting about the role of sarcopenia as a predictor of survival in HCC who underwent TACE (Table 1). Fujita *et al*[59] and Kobayashi *et al*[60] showed no significant association between muscle volume at baseline and clinical outcomes. On the contrary, Loosen *et al*[61] and Dodson *et al*[38] showed that pre-interventional sarcopenia was associated with poor outcomes. Significant heterogeneity was noted in the methods to evaluate sarcopenia in these studies. The total psoas area (TPA), PMI, and L3-SMI were used to evaluate the presence of sarcopenia. If sarcopenia directed these effects (on the TACE efficacy) beyond the patients' general clinical condition or if this is mere an association, needs further evaluation in a prospective fashion. Data on the effects of sarcopenia on HCC patients with TARE is even more limited. Recently, Faron *et al*[32] reported 58 HCC patients using MRI-derived fat-free muscle area (FFMA) to predict sarcopenia. The FFMA < 3582 mm^2 for men and < 2301 mm^2 for women were used. In this study low FFMA was associated with significantly reduced OS (197 *vs* 294, $P = 0.02$)[32].

SURGICAL TREATMENTS

Liver resection

The role of sarcopenia in HCC patients undergoing liver resection is increasingly become topic of interest. Since, HCC patients often have poor nutritional status,

methods to reduce the catabolic state and improve protein synthesis, regeneration, Fan *et al*[62] investigated 124 patients to evaluate the role of nutrition in HCC resection. Nutrition therapy given prior to the liver resection with branched chain amino acids (BCAA), lipids, and dextrose have shown to decrease the worsening of liver function, sepsis-related complications, need for treatment for ascites, and overall decreased mortality. There was a reduction in the overall post-operative morbidity in the nutrition group compared to the control group (34% *vs* 55%; relative risk, 0.66; 95%CI: 0.45-0.96)[62]. In 2013, Harimoto and colleagues[63] studied 186 HCC patients with sarcopenia using L3-SMI (< 43.75 for men, < 41.10 for women), and a significant correlation was noted between sarcopenia and liver dysfunction (indicated by low albumin levels and indocyanine green retention). In patients with and without sarcopenia, the 5-year OS rate was 71% and 83·7%, and the 5-year recurrence-free survival rate was 13% and 33·2%, respectively[63]. Additionally, studies evaluated the relationship between total functional liver volume (TFLV) and sarcopenia (L3-SMI) and found that median TFLV was significantly lower in the sarcopenic group than the normal group (1296 mL *vs* 1840 mL; $P < 0.05$)[64].

Sarcopenic obesity characterized by increased fat volume compared to skeletal muscle mass. As obesity and loss of muscle share common pathophysiological mechanisms, combined insult could display a poor outcome. Studies evaluated the effect of sarcopenic obesity in HCC and found that patients with sarcopenic obesity had worse median survival (84.7 mo *vs* 39.1 mo, $P = 0.002$) and worse median recurrence-free survival (21.4 mo *vs* 8.4 mo, $P = 0.003$)[21]. Additionally, it was identified as an independent risk factor for death and HCC recurrence[21]. Effect of sarcopenia on immediate and short-term clinical outcomes after hepatic resection was examined by Otsuji *et al*[65] Sarcopenic patients had a higher postoperative length of stay, higher rates of liver failure, major complications, and intra-abdominal abscess formation (Table 2). Multiple other studies have provided similar results with different modalities to evaluate sarcopenia, such as L3-SMI, TPA, and visceral-to-subcutaneous adipose tissue ratio (Table 2). Furthermore, these studies used differing SMI cut-off points to define sarcopenia. The majority of the studies point to poor outcomes in patients with sarcopenia, which might be due to the underlying liver dysfunction and HCC severity. Nevertheless, prospective data with uniform cut-off points to assess SMI to define sarcopenia in these studies to provide concrete evidence of the relationship between sarcopenia and liver resection.

Liver transplantation

Sarcopenia in patients awaiting liver transplantation (LT), perioperative and postoperative outcomes have been studied recently[66-71]. Multiple methods to assess sarcopenia were used (Table 3). For example, L3-SMI, psoas muscle thickness, MELD-sarcopenia score, skeletal muscle mass-to-visceral fat area ratio (SVR), TPA, PMA, and height normalized psoas muscle thickness were used. Among these, L3-SMI is the most commonly used objective way of assessing sarcopenia. Further, studies evaluated the wait times and survival related to sarcopenia (Table 3).

Studies performed on outcomes in LT patients evaluated the preoperative status of the patients (listed and waiting for the transplant), procedural outcomes and post-procedure long-term survival. Carey *et al*[68] in 2016 used L3-SMI with 50 cm^2/m^2 for men and 39 cm^2/m^2 for women and noted that individuals who died had lower SMI compared to those who survived (45.6 cm^2/m^2 *vs* 48.5 cm^2/m^2; $P < 0.001$), and SMI was associated with wait-list mortality (HR, 0.95; $P < 0.001$). Wada *et al*[67] in 2017 considered sarcopenia for TPA of 791.6 mm^2/m^2 for men and 488.8 mm^2/m^2 for women. The authors compared TPV to TPA. The preoperative total psoas volume (TPV) was found to be a better predictor than TPA in assessing post-operative risks in living-donor LT recipients[67]. Multiple studies evaluated the LT outcomes and complications such as infections, length of stay, failure to rescue, and surgery-related events[72,73]. The rate of infections was assessed and compared to individuals with sarcopenia. Patients with sarcopenia had a higher prevalence of sepsis, bacterial pneumonia, longer ICU stays, and mortality[2,69]. Postoperative survival was studied by Van Vugt *et al*[69] and Kaido *et al*[72] who noted that sarcopenia was inversely associated with clinical outcomes after LT. Few studies noted sarcopenia developing after the LT, which is probably due to underestimation of muscle mass/strength estimation before LT. In addition to underlying cirrhosis, increased catabolism, tumor-related morbidity noted in these patients, the role of immunosuppressant use cannot be underestimated. The use of mTOR and calcineurin inhibitors can potentially lead to sarcopenia[74]. Further, renal dysfunction caused by calcineurin inhibitors can compound these effects. The results of these studies provide an opportunity for improving the nutritional status in sarcopenia LT patients with dietary and exercise

Table 2 Outcomes of hepatocellular carcinoma patients undergoing liver resection (hepatectomy) with sarcopenia over last 5 years

Ref.	Technique	*n*	Methods and outcomes
Otsuji *et al*[65] (2015, Japan)	Major hepatectomy and extrahepatic bile resection	256	Total psoas area (TPA) was used to assess sarcopenia. TPA of < 567 mm/m^2 for men and < 395 mm/m^2 for woman. Length of postoperative hospital stay were longer (39 d *vs* 30 d, P < 0.001, high rate of liver failure (33% *vs* 16%), major complications (54% *vs* 37%), intra-abdominal abscess (29% *vs* 18% compared to those without sarcopenia (P < 0.05)[69].
Voron *et al*[110] (2015, Japan)	Hepatectomy	198	L3-SMI used 52.4 cm^2/m^2 for men and 38.9 cm^2/m^2 for women. Sarcopenia was associated with shorter median OS (52.3 mo *vs* 70.3 mo; P = 0.01 and it was an independent predictor of OS and DFS.
Yabusaki *et al* [111] (2016, Japan)	Primary hepatectomy	195	SMI used 43.75 cm^2/m^2 for men and 41.10 cm^2/m^2 for women. Sarcopenia was associated with poor cumulative recurrence rate (P = 0.13).
Takagi *et al*[113] (2016, Japan)	Curative hepatectomy	254	L3-SMI used 46.4 cm^2/m^2 for men and 37.6 cm^2/m^2 for women. The sarcopenic group had a significantly lower 5-yr OS rate than the non-sarcopenic group (58.2% *vs* 82.4%, P = 0.0002). Further it was an independent predictor of poor survival (HR =2.28, P = 0.002) and poor ASA status (HR = 3.17, P = 0.001).
Kobayashi *et al* [21] (2019, Japan)	Hepatectomy	465	L3-SMI used. 40.31 cm^2/m^2 for men and 30.88 cm^2/m^2 for women. Sarcopenic obesity as a significant risk factor for mortality (HR = 2.504, P = 0.005) and recurrence of HCC (HR = 2.031, P = 0.006) after hepatectomy for HCC.
Hamaguchi *et al* [112] (2019, Japan)	Hepatectomy	606	L3-SMI was used to assess the sarcopenia. SMI of < 40.31 for men and 30.88 for women were used. A high visceral-to-subcutaneous adipose tissue ratio, low SMI, and high IMAC contributed to an increased risk of death (P < 0.001) and HCC recurrence (P < 0.001) in an additive manner.
Xu *et al*[22] (2020, China)	Hepatectomy	1420	Authors performed a meta-analysis of six studies and preoperative sarcopenia was significantly associated with poor OS (HR =1.58, 95%CI: 1.34-1.84, P = 0) and shorter DFS (HR =1.54, 95%CI: 1.17-2.02, P = 0.002) in patients with HCC undergoing hepatectomy[24].

Studies, techniques and outcomes to evaluate the success of liver resection in patients with sarcopenia and hepatocellular carcinoma. L3-SMI: Third lumbar vertebrae- skeletal muscle Index; OS: Overall survival; SMI: Skeletal muscle index; HR: Hazards ratio; DFS: Disease free survival. HCC: Hepatocellular carcinoma; TPA: Total psoas area; IMAC: Intramuscular adipose tissue content; PMI: Psoas muscle index.

measures during pre, peri and post-operative period.

Systemic therapies

The use of chemotherapy and immunotherapy has become the mainstay of treatment for HCC lesions that are not amenable to LRT or LT. Sorafenib is the most studied and prescribed chemotherapeutic agent in HCC[75]. Although it can prolong survival, its use is limited by its adverse effects such as nausea, excessive fatigue, and diarrhea noted in most patients. These studies evaluated multiple outcomes such as OS, progression-free survival, mortality were evaluated in different studies in HCC patients receiving Sorafenib therapy[76-82]. While the ways to assess the sarcopenia differed in these studies, most commonly used method is L3-SMI. Further various cut-off values were utilized in these studies.

Nishikawa *et al*[78] studied 232 patients to evaluate for OS using L3-SMI. The authors noted that the patients with sarcopenia had significantly low median OS of 174 d compared to 454 d in the non-sarcopenic group (P < 0.0001). Multivariate analysis showed that sarcopenia was an independent predictor of OS. Similarly, Takada *et al*[81] studied 214 patients in which OS in pre-sarcopenia patients were worse than without pre-sarcopenia (median 252 d *vs* 284 d, respectively; P = 0.16). Saeki *et al*[82] reported 100 advanced HCC patients using use of L3-SMI showing individuals without muscle depletion had longer survival was noted (HR = 0.50, P = 0.006). This combined with low tumor number (< 7) and lack of extrahepatic spread offered better survival in these patients[82]. Dynamic assessment of sarcopenia has assisted to compare outcomes before and after starting sorafenib. Few studies noted that sarcopenia worsened after the initiation of sorafenib. If this is due to the progression of HCC or angiogenic (or Carnitine inhibitory) properties of sorafenib needs further evaluation[83]. Further, Cheng *et al*[34] reported that pre-sarcopenia could independently predict the outcomes in sorafenib-failed HCC.

Use of other modalities such as fat mass indices (visceral, subcutaneous) in combination with L3-SMI and their relative changes (over a period of time) can assist in assessing sarcopenia and can predict outcomes in HCC patients receiving sorafenib [82]. However, more studies are needed to confirm these findings. Recently newer agents for HCC are increasingly utilized such as Regorafenib, Lenvatinib, Nivolumab, the combination of gemcitabine and oxaliplatin (GEMOX regimen)[30,84-86]. Studies showing the effect of sarcopenia on HCC patients' survival using these agent are

Table 3 Outcomes of hepatocellular carcinoma patients undergoing liver transplant with sarcopenia over last 5 years

Ref.	Technique	*n*	Methods and outcomes
Itoh *et al*[114] (2016, Japan)	Living-donor LT	153	Based on SVR, patients with low SVR were had poor prognosis than without low SVR for OS (P = 0.03) and recurrence-free survival (P = 0.01).
Carey *et al*[68] (2016, United States)	Awaiting LT	396	L3-SMI used. 50 cm^2/m^2 for men and 39 cm^2/m^2 for women. Patients who died had lower SMI compared to those who survived (45.6 cm^2/m^2 *vs* 48.5 cm^2/m^2; P < 0.001), and SMI was associated with wait-list mortality (HR, 0.95; P < 0.001)[72].
Wada *et al*[67] (2017, Japan)	LDLT	32	TPA was used. TPA of 791.6 mm^2/m^2 for men and 488.8 mm^2/m^2 for women. TPV was used to compare to TPA. Preoperative TPV is a better predictor compared to TPA in assessing post-operative risks in LDLT recipients[71].
Golse *et al*[70] (2017, Europe)	LT	256	PMA, L3-SMI was used. 1561 mm^2 for men and 1464 mm^2 for women. One and 5-yr OS rates were significantly poorer in the sarcopenic group than in the nonsarcopenic group at 59% *vs* 94% and 54% *vs* 80%, respectively (P < 0.001). Authors concluded that pre-LT PMA might be predict 1-yr survival post-LT[74].
Van Vugt *et al*[69] (2017, Europe)	Listed for LT	585	L3-SMI used. 43 to 53 cm^2/m^2 for men based on the BMI and 41 cm^2/m^2 for women. Sarcopenia was associated with waiting list mortality in liver transplant candidates with cirrhosis, particularly in patients with lower MELD scores (P < 0.001) [73].
Kim *et al*[71] (2018, Japan)	LDLT	92	Height normalized psoas muscle thickness (< 15.5 mm/m) at L3. HCC recurrence risk was greater in sarcopenic patients in univariable analysis [HR = 8.06 (1.06–16.70), P = 0.044) and in multivariable analysis [HR = 9.49 (1.18–76.32), P = 0.034][75].
Chae *et al*[66] (2018, South Korea)	LDLT	408	This study investigated the association between a perioperative decrease in the PMI and patient mortality after LT. A PMI decrease ≤-11.7% between the day before surgery and POD-7 was an independent predictor of patient mortality after LT[70].

Techniques, methods and outcomes to evaluate the success of liver transplantation in patients with sarcopenia and hepatocellular carcinoma. LT: Liver transplant; LDLT: Living-donor LT; SVR: Skeletal muscle mass-to-Visceral fat area ratio; TPV: Total psoas volume; PMA: Psoas muscle area; BMI: Body mass index; L3-SMI: Third lumbar vertebrae- skeletal muscle index; OS: Overall survival; SMI: Skeletal muscle index; HR: Hazards ratio; DFS: Disease free survival; HCC: Hepatocellular carcinoma; TPA: Total psoas area; PMI: Psoas muscle index.

sparse. Lenvatinib induces minimal muscle loss after 2 years of treatment correlates with its low toxicity[23,30,87]. Combined effects of sarcopenia and inflammation (by high neutrophil-to-lymphocyte ratio and absolute lymphocyte count) have been studied in patients receiving nivolumab in HCC patients[28]. If inflammatory markers are more important than sarcopenia in patients received immunotherapy needs further validation[25,28]. Overall, sarcopenia can predict survival in advanced HCC patients receiving chemotherapeutics such as sorafenib before initiation of the treatment and during and after the treatment. Strategies to improve the muscle mass, nutrition can add to the survival in these patients. Further studies are needed to evaluate the role of sarcopenia for new chemotherapy and for immunotherapy.

METHODS TO IMPROVE SARCOPENIA

As sarcopenia can adversely affect the outcomes of HCC patients undergoing treatments, methods to improve could impact the survival of these patients. As HCC happens with a background of cirrhosis in up to 80%-90% of patients, improving sarcopenia in cirrhotics could assist in improving survival. Reversing pathophysiology by improving myofibres size, number, reversing myosteatosis, inhibiting mitochondrial integrity loss, mTOR signaling, and decreasing ROS accumulation can improve sarcopenia in both HCC and cirrhotics. Two major strategies exist to improve sarcopenia in these patients- nutritional support and physical exercise. Use of L-carnitine, BCAA, leucine have been used in the studies to increase the nutritional component[88,89]. Improvement of skeletal mass (PMI) was noted after the supplementation of these agents in these studies. Physical exercise can recruit more myofibres and at least inhibit sarcopenia. It is unclear if it can reverse the sarcopenia completely. Both isometric (lifting hand weights 2-3 times per week) and isotonic (30-40 min walking 3-4 times per week) have been used to improve muscle strength in these patients[90-94]. Studies have shown an increased muscle cross-sectional area (quadriceps) with exercise in cirrhotics of at least 10%[95]. Although, testosterone supplementation have been reported to improve the sarcopenia, few reports of alpha-alkylated formulation could theoretically increase the risk of HCC formation[96].

The role of non-steroidal Selective Androgen Receptor Modulators (SARMs) is increasingly being recognized in the treatment of sarcopenia[97-99]. SARMs inhibit protein degradation and thereby could decrease the rate of sarcopenia. Multiple animal models have been used to evaluate mechanisms of SARMs to reverse muscle atrophy in degonadized mice. For instance, SARM treatment in ovariectomized rat model can increase muscle mass by enhanced mitochondrial biogenesis, actin and myosin[98]. SARMs can target androgen receptors and decrease sarcopenia *via* paracrine growth factor signaling on vimentin positive muscle fibroblasts[97]. Further, upregulation of mTOR, glycogen synthase kinase[99]. SARMs also exhibit anabolic effects, increasing the bone and muscle mass which are affected in patients with HCC. A combination approach of nutritional supplementation with physical exercise with a multidisciplinary approach has been tried in cirrhotics and HCC patients[31]. Significant changes in muscle volume was noted after the intervention[95]. Similarly, a combined approach has been tried in a few studies in HCC patients undergoing TACE [100,101]. This approach has been studied in patients waiting or LT, with good response[102,103]. In conclusion, a combined multidisciplinary approach is useful and logical to improve the sarcopenia in cirrhotics and HCC which might eventually improve outcomes of these patients undergoing local, surgical and systemic therapies.

FUTURE DIRECTIONS

Although sarcopenia can offer significant details about the functional status, it can be further enhanced by the use of frailty (using clinical frailty scale, liver frailty index, Karnofsky performance status) and amount of malnutrition (by assessment of BMI, nutritional intake). These can be incorporated into composite scoring to better evaluate the functional status of HCC patients. Recently use of changes in bone resorption *via* upregulation of inflammatory cytokines opened the concept of sarcopenic osteoporosis [104]. A crosstalk between skeletal muscle, bone homeostatic changes with underlying cirrhosis and HCC can provide pathways for treatments in the future. Myostatin, irisin, osteocalcin, activation of Wnt/β-catenin pathways have been implicated in sarcopenic osteoporosis. Furthermore, biomarkers such as imbalance of plasma free amino acids (BCAA) have been implicated in progression of HCC[105]. If this could be a reliable way to improve the sarcopenia in HCC patients remains to be studied.

Precision medicine tools such as use of radiomics and radiogenomics are emerging for assessing host and tumor-related risk factors in HCC[106,107]. Radiomics uses medical imaging data to develop reproducible quantitative data from qualitative images. This has been utilized for lung cancer assessment of tumor and non-tumor tissue[108]. Development of methods to quantify the amount of normal non-tumor liver tissue in HCC patients is essential for surgeons to evaluate resection strategies. Seror *et al*[109] noted that use of non-invasive cross-sectional imaging to assess the liver surface nodularity and lean body mass can act as surrogate markers for liver cirrhosis and sarcopenia. Patients with higher liver surface nodularity (OR 7.05, 95%CI: 2.13-23.25) and sarcopenia (OR 6.51, 95%CI: 2.08-20.39) were associated with high risk of complications[109]. A step further in this direction, use of genomics (cellular and molecular changes) to existing radiomics can provide radiogenomic information which can be used to develop molecular signatures for development for actionable clinical targets[107]. Finally use of artificial intelligence and deep learning can lead to next generation biostatistical and informatic data to develop algorithms and pathways to identify optimal clinical patterns[106].

CONCLUSION

Sarcopenia is increasingly recognized as a predictive marker for assessing outcomes in HCC patients. There is increasing evidence to evaluate its role in loco-regional, surgical, transplant, and systemic treatment options in HCC patients. Early recognition to identify sarcopenia, methods to improve the muscle volume, strength, and mass could impact the patient outcome and OS. The use of appropriate nutritional support, physical activity or both could potentially improve muscle volume in these patients. However, it is unclear about the degree of improvement of the sarcopenia with all of these measurement combined. Further, prospective studies aimed at interventions that could potentially reverse sarcopenia to improve HCC patients' outcomes are needed in the future.

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MINIREVIEWS

Celiac disease: From genetics to epigenetics

Elisa Gnodi, Raffaella Meneveri, Donatella Barisani

ORCID number: Elisa Gnodi 0000-0002-4390-6633; Raffaella Meneveri 0000-0003-0791-5951; Donatella Barisani 0000-0003-4592-6221.

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Elisa Gnodi, Raffaella Meneveri, Donatella Barisani, School of Medicine and Surgery, University of Milano-Bicocca, Monza 20900, Italy

Corresponding author: Donatella Barisani, MD, MSc, Associate Professor, School of Medicine and Surgery, University of Milano-Bicocca, Via Cadore 48, Monza 20900, Italy. donatella.barisani@unimib.it

Abstract

Celiac disease (CeD) is a multifactorial autoimmune disorder spread worldwide. The exposure to gluten, a protein found in cereals like wheat, barley and rye, is the main environmental factor involved in its pathogenesis. Even if the genetic predisposition represented by HLA-DQ2 or HLA-DQ8 haplotypes is widely recognised as mandatory for CeD development, it is not enough to explain the total predisposition for the disease. Furthermore, the onset of CeD comprehend a wide spectrum of symptoms, that often leads to a delay in CeD diagnosis. To overcome this deficiency and help detecting people with increased risk for CeD, also clarifying CeD traits linked to disease familiarity, different studies have tried to make light on other predisposing elements. These were in many cases genetic variants shared with other autoimmune diseases. Since inherited traits can be regulated by epigenetic modifications, also induced by environmental factors, the most recent studies focused on the potential involvement of epigenetics in CeD. Epigenetic factors can in fact modulate gene expression with many mechanisms, generating more or less stable changes in gene expression without affecting the DNA sequence. Here we analyze the different epigenetic modifications in CeD, in particular DNA methylation, histone modifications, non-coding RNAs and RNA methylation. Special attention is dedicated to the additional predispositions to CeD, the involvement of epigenetics in developing CeD complications, the pathogenic pathways modulated by epigenetic factors such as microRNAs and the potential use of epigenetic profiling as biomarker to discriminate different classes of patients.

Key Words: Celiac disease; Epigenetics; DNA methylation; Histone modifications; Long non-coding RNAs; MicroRNAs

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Core Tip: Currently identified genes account only for half of celiac disease (CeD)

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predisposition. An important role could be played by epigenetics, inheritable traits without DNA sequence alterations, which could be influenced by gluten exposure. DNA methylation, histone modifications and non-coding RNAs act on different gene expression steps, from gene transcription to post-translational ones. Epigenetic changes can be additional predisposition factors or specific of CeD stages (active disease, gluten-free diet) as recently reported. Analysis of epigenetic data and their integration with transcriptome (by machine learning) can help to stratify patients, or discover new players in CeD pathogenesis, possible focus of novel therapeutic approaches.

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INTRODUCTION

Celiac disease (CeD) is a multifactorial autoimmune enteropathy that develops in genetically predisposed subjects carrying the HLA-DQ2 or HLA-DQ8 haplotype. The prevalence of these HLA haplotypes in the general population is around 30%-40%, suggesting that they are necessary, but not sufficient, to induce CeD[1]. Regional variancies have been observed in the frequency of these haplotypes in CeD, with the HLA-DQ8 that ranges from about 2%-4% in western countries to 25%-30% in the Iranian population and in the middle East[2]. Indeed, CeD prevalence worldwide is about 1%-2%, with environmental factors that contribute to the regional differences, like the exposure to gluten, the known CeD exogenous antigen[1]. The clinical presentation of CeD is quite heterogeneous, ranging from classical intestinal-related symptoms (diarrhoea, failure to thrive) to non-intestinal manifestations (anaemia, dermatitis, osteoporosis), and such diverse clinical picture may delay, in some cases, the prompt diagnosis[3]. Even if the main mechanisms by which gluten peptides cause CeD intestinal lesion are now quite established, the reason why only a few of the genetically predisposed subjects develop the disease still needs to be clarified. In fact, geneticists calculated that the presence of a specific HLA accounted only for about 40% of the genetic predisposition, leaving most of the genes involved in the development of the disorder still unknown[4]. Thus the initial focus of researchers was to identify further genes that could constitute a "genetic background" predisposing to the disease. Few studies used the classical linkage analysis approach, but the obtained results were specific of a single population and could not be replicated[5], or the identified genomic region did not harbour genes that appeared to be involved in CeD pathogenesis[6]. The identification of additional predisposing genes was obtained using a different approach, *i.e.*, Genome Wide Association Studies (GWAS) performed on populations of different geographical origin. These studies identified 39 Loci associated with CeD development, but also confirmed the role of the Human Leukocyte Antigen (HLA) region. Additional analyses revealed that some loci included more than one gene associated with CeD, thus raising the number of the involved polymorphisms (single nucleotide polymorphisms, SNPs) up to 57. Although these studies were quite extensive, the identified loci were not able to completely explain CeD genetic predisposition, since HLA + all these additional genes accounted for about 54% of the hereditability of CeD[7]. Interestingly, a great number of these SNPs involved genes with an immune function either in the intestine or in the thymus, further supporting the idea that an alteration of the immune response represents an essential step in CeD predisposition and pathogenesis. Moreover, a more recent paper was able, integrating genomic and transcriptomic data, to prioritize genes involved in CeD, and to identify TRAF-type zinc finger domain containing 1 (TRAFD1) as a master regulator of genes involved in interferon (IFN)γ signaling and MHC I antigen processing/presentation[8]. The different combination of these SNPs could also be associated with a different phenotype, as recently reported by Cerquieira *et al*[9], who identified the TLR7/ TLR8 Locus associated with disease onset before 7 years of age, whereas SH2B3/ATXN2, ITGA4/UBE2E3 and IL2/IL21 Loci were associated with later development of CeD and a more severe small bowel mucosal damage. In addition, SH2B3/ATXN2 was associated with type 1 diabetes; in fact some of the

identified SNPs are described as predisposing factors also for other autoimmune disorders like type 1 diabetes and Crohn's disease[7,10], almost suggesting the presence of a common genetic background predisposing to autoimmunity. It must also be noted that, among the identified SNPs, only 5% were present in coding regions, and about 5% and 9% in 5' and 3' untranslated regions, respectively[7]. This means that 81% of the identified SNPs were either in intergenic or intronic regions, suggesting that their role could be to regulate gene expression, possibly through the interaction with transcription factors or proteins able to regulate chromatin status, *i.e.*, epigenetic modifications. Epigenetics has gained much attention in recent years, but its involvement in CeD still needs to be well characterized. First of all, this term has been used to define a wide range of concepts, and the 2008 Cold Spring Harbor meeting defined epigenetics as *"the study of a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"*[11]. DNA methylations, histone modifications resulting in chromatin remodelling and non-coding RNAs are the three main categories that fall into this statement. The latter one also includes microRNAs, that have been found deregulated in CeD patients, suggesting that the regulation of the expression of target mRNAs by post-transcriptional modifications is relevant in CeD pathogenesis[12-14]. In this direction, RNA sequencing studies are currently trying to identify different gene expression signatures that could help stratify patients based on CeD stage/presentation, or highlight new pathways implicated in CeD development[15-17].

In this review we will provide an update on the current knowledge about epigenetics in CeD and investigate the possible role of epigenetic profiling in patient classification and/or in determining the risk in subjects with familiarity to CeD.

LITERATURE SEARCH

The literature search was carried out on PubMed, searching the key words "epigenetics", "histones -acetylation, -methylation, -phosphorylation, -ubiquitination", "chromatine", "non-coding RNA", "lncRNA", "miRNA", "microRNA", "RNA methylation" AND "celiac disease". The reviewed papers were original research articles published from 2010 to October 2021. All articles that were considered relevant to the purpose of the present review were included, whereas the ones that did not add novelty or did not give clear results were excluded during the critical revision of the literature.

DNA METHYLATION

DNA methylation is the most known epigenetic modification, that sees the family of DNA methyltransferase (DNMTs) enzymes transfer methyl-groups to the C-5 residue of cytosine pyrimidine ring. This phenomenon occurs in particular in CpG-rich regions (CpG islands), mainly localized in promoters and regulatory regions. Being the methylated DNA physically less accessible for transcription, this causes the inactivation of the nearby genes[18]. The majority of gene promoters are normally methylated to guarantee a fine regulation of transcription in different tissues or cell types. Like most of the epigenetic modifications, it does not directly alter the DNA sequence, but a typical methylation profile can usually be inherited. Hypo-methylation or hyper-methylation of specific genomic loci has been reported to predispose to disease and cancer[19]; for this reason, the first studies on DNA methylation in CeD analyzed the predisposition to develop small bowel adenocarcinomas. In particular, Bergmann *et al*[20] found a high level of CpGs methylation and microsatellite instability correlated to the loss of MLH1 expression in three different small bowel carcinomas in CeD patients, whereas this feature was not present in non-CeD patients. A similar finding was observed by Diosdado *et al*[21], who also detected an hyper-methylation of the *APC* gene promoter that caused defects in the mismatch repair mechanisms in these patients. Further studies were conducted by Rizzo *et al*[22], who were able to identify four different CpG Island Methylator Phenotypes, with two of them being specific for CeD patients.

Although these data highlight a typical methylation pattern in CeD complications, the identification of a methylation profile associated with the predisposition for developing CeD is essential. Since GWAS pointed out the presence of many risk variants common to other autoimmune diseases, it has been hypothesised that they could also share common methylation patterns. Hammaker *et al*[23] focused on the

specific risk variant rs906868, shared between CeD and rheumatoid arthritis and mapping in the promoter of LBH, gene with a regulatory function on the Wnt pathway, essential for differentiation and development. Interestingly, the authors detected a differential methylation pattern in the two diseases, thus suggesting the possibility of a disease-specific profile[23]. Methylation can also occur only on one allele, thus not causing a complete silencing of the gene but its modulation. Hutchinson *et al*[24] investigated allele-specific methylation (ASM) on CpG islands localized throughout the genome, detecting four of them as implicated in complex diseases. In particular, rs2762051, a C/T risk variant that maps in the non-coding RNA DLEU1, undergoes ASM in CeD[24]. It is interesting to note that this variant belongs to the group of SNPs previously identified as significantly associated to CeD predisposition[7], thus further supporting the need for an integration between the genetic and epigenetic profile. A specific gene methylation could also be associated to a different phenotype, in particular if the methylation site is able to influence the expression of disease-related proteins. In fact, a lower level of methylation was detected in different IFNγ CpGs in patients with ulcerative colitis (UC) and a more severe phenotype[25], suggesting that methylation can regulate this UC pivotal pathogenetic pathway. Fernandez-Jimenez *et al*[26] demonstrated that this can also happen in CeD, since they observed not only a different methylation profile in the genes of the core NFkB pathway in active CeD subjects as compared to controls, but also an inter-mediate pattern in CeD patients on gluten-free diet (GFD). Although it was not clear if gliadin could directly affect the methylation of these specific genes, long exposure to gliadin could be able to affect the methylation pattern, since proline-rich peptides seem to act as opioid-like molecules causing a modulation in glutathione activity and DNA methylation[27].

It is also known that methylation profiles can be cell type-specific, thus a single cell methylation analysis could provide essential information. However, the methods employed to obtain single cell preparations can sometimes alter the DNA methylation patterns as described by Jenke *et al*[28], thus pointing out the importance of considering this aspect in designing this kind of studies. Another potential bias of methylation studies regards the preferential amplification of an allele or a strand, based on its methylation degree. For this reason Ochoa *et al*[29] realized a new Bayesan calibration method and validated it also on CeD patients samples, and the most recent studies on methylation in CeD considered these techniques. Fernandez-Jimenez *et al*[30] compared the whole methylome of the epithelium and immune cells from CeD and controls biopsies, finding a cell-specific methylation pattern, with 43 (11 hypo-, 32 hyper-methylated) and 310 (40 hypo-, 270 hyper-methylated) differentially methylated positions (DMP) in the epithelium and in immune cells respectively. It is important to note that only a portion of the DMPs was shared between the two cellular components, highlighting a cell-specific, disease-driven modification. Cielo *et al*[31] used a different approach, separating the epithelium from the lamina propria and analysing the expression and methylation of genes known to be altered in CeD. Among the candidate genes, they found a decreased methylation in SB2H3 and IL21, that coupled with their increased expression in the epithelium and lamina propria respectively, but only in active CeD compared to controls. Since these genes take part in the pathways mainly involved in inflammation and barrier integrity in CeD, identifying a different signature in patients and controls may partially explain the abnormal response to food antigens and help patient stratification[31]. Finally, the most recent study in this field by Hearn *et al*[32] compared methylation profiles in saliva samples obtained from CeD patients on a GFD and controls with the use of a beadchip array, finding a different methylation pattern in HLA-DQB1 and in specific loci near *SLC17A3* gene in a pilot cohort, data not confirmed in a larger study group. Being this collection method non-invasive, it could be a great tool for patient discrimination, especially in the screening of predisposed subjects, once a different and discriminatory methylation profile has been clearly established. Moreover, the patient histological classification needs to be taken into account, even if a partial concordance in methylation patterns in saliva and intestine has been observed in a small group of patients[33]. In this direction, encouraging results come from studies on inflammatory bowel diseases (IBDs) from Howell *et al*[34], that were able to statistically discriminate different classes of patients by analysing the transcriptome and the methylation status in the intestinal epithelium, correlating them with the disease outcome. Indeed, methylation profiling can be an asset to study different aspects of CeD, both to understand its pathogenesis and as a biomarker. A graphical representation of DNA methylation is present in Figure 1, whereas its highlights in CeD are reported in Table 1.

Table 1 DNA methylation features in celiac disease

Predisposition to CeD	What	Result	Highlight in CeD
Allele-specific methylation (ASM) [24]		ASM in rs2762051 in *DLEU1* gene	Linked to CeD phenotype
Rs906868 in *LBH* gene promoter [23]	Risk variant shared with RA	Disease-specific methylation	Different methylation in rs906868 can predispose to CeD or RA → influence on Wnt signalling
Methylation in HLA region in CeD[30]	Specific patterns in epithelial and immune cells	Genotype-independent methylation (except for HLA-DPB2)	Methylation patterns in HLA region → CeD predisposition
Methylation profiling in HLA-DQB1 and SLC17A3[32]	Bead-chip on saliva samples	Different methylation profiles, not confirmed in bigger cohort	Potential methylation-based screening Major validation needed
Opioid like-effect of gliadin[27]		Modulation of glutathione and DNA methylation	Predisposition to inflammation and oxidation
CeD pathogenesis			
Methylation in NFkB-related genes[26]	NFkB pathway↑↑	Disruption of regulatory equilibrium	Co-methylation patterns typical of active CeD in NFkB pathway genes
Cell-specific methylation[30]		Epithelium → 43 DMP Immune cells → 310 DMP	Cell-specific methylation signature & gene expression in CeD *vs* controls
Different methylation of SB2H3, IL-21, cREL and TNFAIP3[31]	Epithelium and lamina propria - specificity	Correlation with pro-inflammatory(↑) and cell adhesion(↓) pathways	Methylation of SB2H3 → epithelium Methylation of IL21 → lamina propria Typical of CeD samples.
Tumor development			
↑CpGs methylation[20]	Microsatellite instability	↓MLH1 expression	Typical in CeD-related small bowel adenocarcinoma
MLH1 deregulation[21]	*APC* gene hyper-methylation	Chromosomal aberrations/microsatellite instability	Defects in mismatch repair Typical in CeD-related small bowel adenocarcinomas
Microsatellite instability[22]	Typical CpGs methylator phenotype	Methylation profiling → phenotypical classification	Mesenchymal and immune phenotypes are common in CeD-related small bowel carcinoma

CeD: Celiac disease; ASM: Allele specific methylation; RA: Rheumatoid arthritis; DMP: Differentially methylated positions; CpGs: CpG islands.

HISTONE MODIFICATIONS

A further step in gene expression regulation in the nucleus is represented by histone modifications. Histones are key proteins of chromatin, able to modify its structure making the DNA more or less accessible to transcription. The most common histone modifications are acetylation, phosphorylation, ubiquitination and methylation. Gene expression analysis studies performed on CeD biopsies compared to controls, pointed out a differential regulation of histone-modifying enzymes, thus suggesting the presence of a disease-related epigenetic signature involving histone modifications[16, 17].

Histone acetylation, governed by acetyltransferase (HATs) and deacetylase (HDACs) enzymes, usually result in active gene transcription due to chromatin relaxation[35]. Zorro *et al*[36] analyzed the transcriptomic and epigenetic responses to IL-15, IFNβ and IL-21 stimulation in primary cytotoxic T lymphocytes derived from CeD biopsies. Specific transcriptomic patterns were identified for the different cytokine stimulation, but also different levels of histone acetylation (H3K27ac) were detected. Interestingly, the increase in transcription after IFNβ stimulation was associated, in about 60% of the cases, with an increase in H3K27ac in promoter and enhancer regions, whereas this was not the case after IL-15 stimulation, pointing towards the presence of a different regulatory mechanism. In fact, upon IL-15 stimulation a relevant number of differentially expressed genes were ncRNAs, suggesting their potential regulatory role independently from H3K27ac modifications

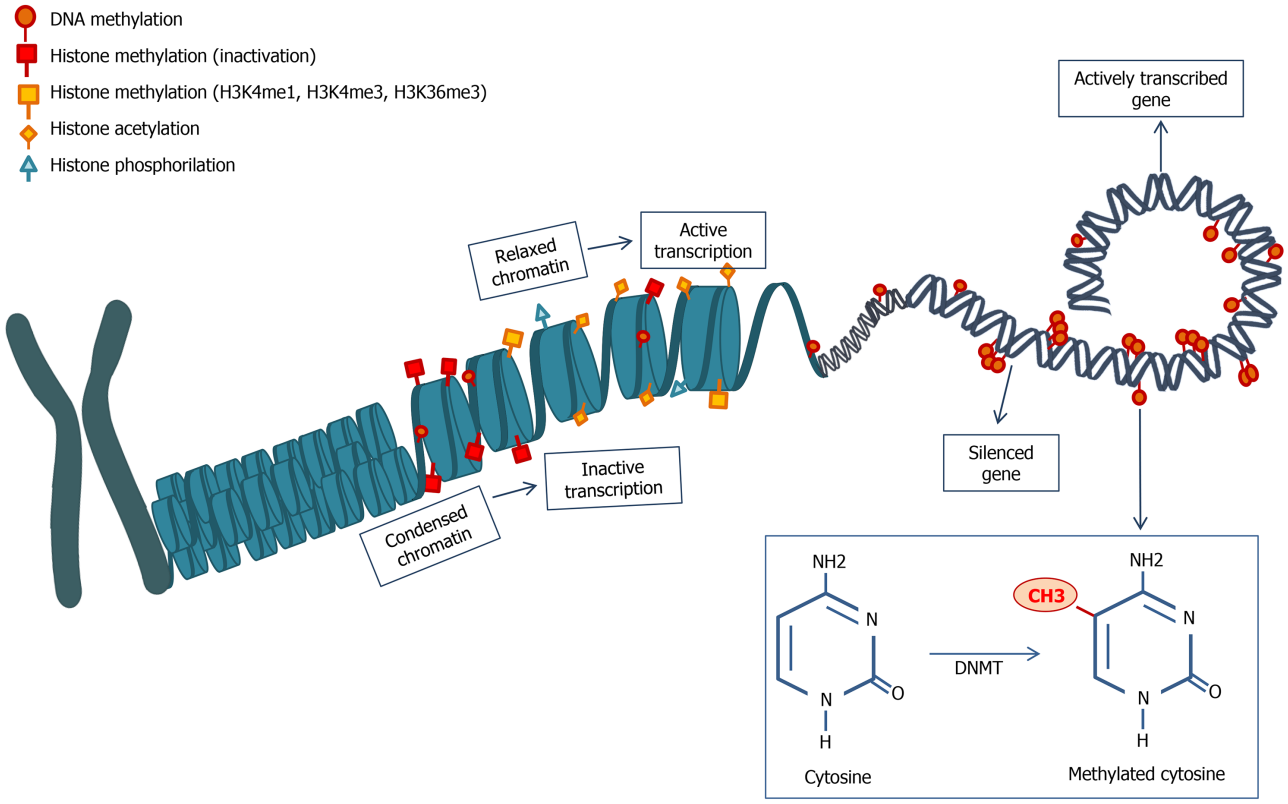


Figure 1 Schematic representation of DNA methylation and histone modifications. Histone modifications are many and determine a different chromatin status, modifying DNA accessibility and interfering with gene transcription. Methylation can also happen directly on the DNA sequence, mainly resulting in gene silencing. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). DNMT: DNA methyltransferase.

[36].

Histone methylation is more complex and mainly linked to repression, but the specific methylations H3K4me1, H3K4me3 and H3K36me3 are commonly found in actively transcribed regions[35]. In fact, H3K4me3 was used as a marker of active transcription by Gutierrez-Achury *et al*[37]; the authors firstly identified SNPs associated with both CeD and rheumatoid arthritis, but also SNPs specific for each disease. The combination of these data with H3K4me3 profiles available in public databases allowed the authors to detect similar histone enrichment corresponding to the common SNPs in less specialized immune cell types, whereas disease-specific SNPs overlapped with H3K4me3 profiles in more specialized cells[37]. H3K36me3 signature was investigated by Moffitt *et al*[38] in the enteropathy associated T cell lymphoma (EATL), the most common neoplastic consequence of CeD. The histone-lysine N-methyltransferase SETD2 is the responsible for this kind of methylation and it was silenced in 32% of EATL in CeD subjects. In fact, *in vivo* studies with a knock out model for SETD2 in T cells carried a decreased H3K36me3 pattern along with γδ-T cells expansion[38]. H3K27me3 was instead used as gene silencing signature by Oittinen *et al*[39] in determining the involvement of the polycomb repressive complex 2 (PRC2) in controlling Wnt signalling in the intestine. They found a H3K27me3 pattern which differed according to the cell position/differentiation along the crypt/villus axis, and involved also genes related to proliferation and differentiation in the epithelium. Thus PRC2-driven tri-methylation is important to maintain the homeostasis driven by Wnt; however, since Wnt pathway deregulation has been connected to CeD pathogenesis, they also hypothesise that a Wnt/PRC2 disrupted axis could cause the development of crypts hyperplasia in CeD[39] (Table 2). However, among the external factors that can influence the epigenetic regulation, the interaction with the microbiota needs also to be considered, since changes in the gut bacterial populations can affect the activity of enzymes involved in epigenetic regulation[40,41].

Table 2 Celiac disease-relevant histone modifications

Modification		Focus	Result	Relevance in CeD
H3K27ac [36]	Activation and enhancing of transcription	Profiling in stimulated CTLs from CeD subjects	IL-15, IFNβ and IL-21 induce specific acetylation profiles in CTLs	Strong association between H3K27ac and gene expression IFNβ-induced IL-15-derived changes related to lncRNAs
H3K4me3 [37]	Active transcription marker	Shared variants between CeD and RA	Similar histone enrichment in shared variants in simple cells	Different histone enrichment in disease-related specialized cells
H3K36me3 [38]	Active transcription marker	SETD2 silenced in 32% of EATL	↓H3K36me3 → γδT cells expansion	Predisposition to lymphomagenesis
H3K27me3 [39]	Gene silencing	PRC2–driven trimethylation	Villi → H3K27me3 on proliferation and differentiation genes Crypts → H3K27me3 on nutrient transport and cell killing genes	PRC2 methylation help maintenance of Wnt homeostasis → deregulation linked to CeD crypts hyperplasia

CeD: Celiac disease; CTLs: Cytotoxic T lymphocytes; LncRNAs: Long non-coding RNAs; ECM: Extracellular matrix; RA: Rheumatoid arthritis; EATL: Enteropathy associated T cell lymphoma; PRC2: Polycomb repressive complex 2.

LONG NON-CODING RNAS

Long non-coding RNAs (lncRNAs) have emerged in recent years as a class of transcripts with a wide spectrum of mechanisms of action that can affect gene expression regulation both at transcriptional and post-transcriptional level, in the nucleus and in the cytosol. Their genomic location is various, either being intergenic or within the introns of coding genes. Among their many mechanisms of action, the most frequent are direct DNA sequence interaction, transcription factor sequestration, chromatin rearrangement, regulation of histone modifications and microRNA sponging (Figure 2)[42,43]. Being implicated in so many regulatory patterns, they have been studied in cancer and in many other diseases, also with an autoimmune background[44].

The pathogenesis of CeD involves several different mechanisms, *i.e.*, the passage of the gluten peptides across the intestinal barrier, but also the activation of innate and adaptive immune response. LncRNAs can influence all these processes, but the expression of these RNAs can, in turn, be affected by the presence of SNPs.

A typical aspect in CeD pathogenesis is the loosening of the tight junctions (TJ), that increases the intestinal permeability[45]. Jauregi-Miguel *et al*[46] identified, in a genomic region associated with CeD, a lncRNA named RP4-587D13.2. This is located in an intron of *MAGI2* gene, a scaffold protein present in the tight junction plaque and able to regulate epithelial integrity. Interestingly, RP4-587D13.2 can regulate MAGI2 expression, altering the downstream TJ-related proteins like CLDN1 and ZAK. RP4-587D13.2 and MAGI2 resulted also downregulated in CeD samples (both with active CeD and on a GFD) and it seems that gliadin stimulation can reinforce this effects[46].

Ricaño-Ponce *et al*[47] identified genes located in close proximity to autoimmune-related SNPs, revealing that 42 of these SNPs could specifically affect the expression of 53 non-coding RNAs. In particular, two lncRNAs were associated to CeD-specific SNPs, namely AP002954.4 and AC104820.2. In both cases the SNPs were able to affect the expression of these lncRNAs, which had a role in the immune response[47]. Interestingly, AC104820.2 had already been detected upregulated in active CeD patients intestinal mucosa by Plaza-Izurieta *et al*[48], who also identified the SNP rs1018326 as associated with this lncRNA. These data thus support the involvement of lncRNAs in the immune response in CeD, however there are aspects that must be kept in mind, as also reported by Hrdlickova *et al*[49]. In fact, although the authors suggested the lncRNAs RP3-395 M20.9 and IL21-AS to be relevant in CeD, and linked them to TNF and IL-21 pathways, they also reported that the same lncRNAs are involved in different autoimmune disorders. In addition, some lncRNAs were detected only in some specific cell subtypes, underlying the need for a cell-specific analysis[49].

Castellanos-Rubio *et al*[50] identified lnc13 as associated to the susceptibility to celiac disease, demonstrating also the functional role of this lncRNA. Lnc13 binds the nuclear ribonucleoprotein hnRNPD and histone deacetylase 1, forming a complex that acts as a repressor of inflammatory gene expression. The presence of inflammatory stimuli activate the NFkB pathway that reduces lnc13, thus removing the repression

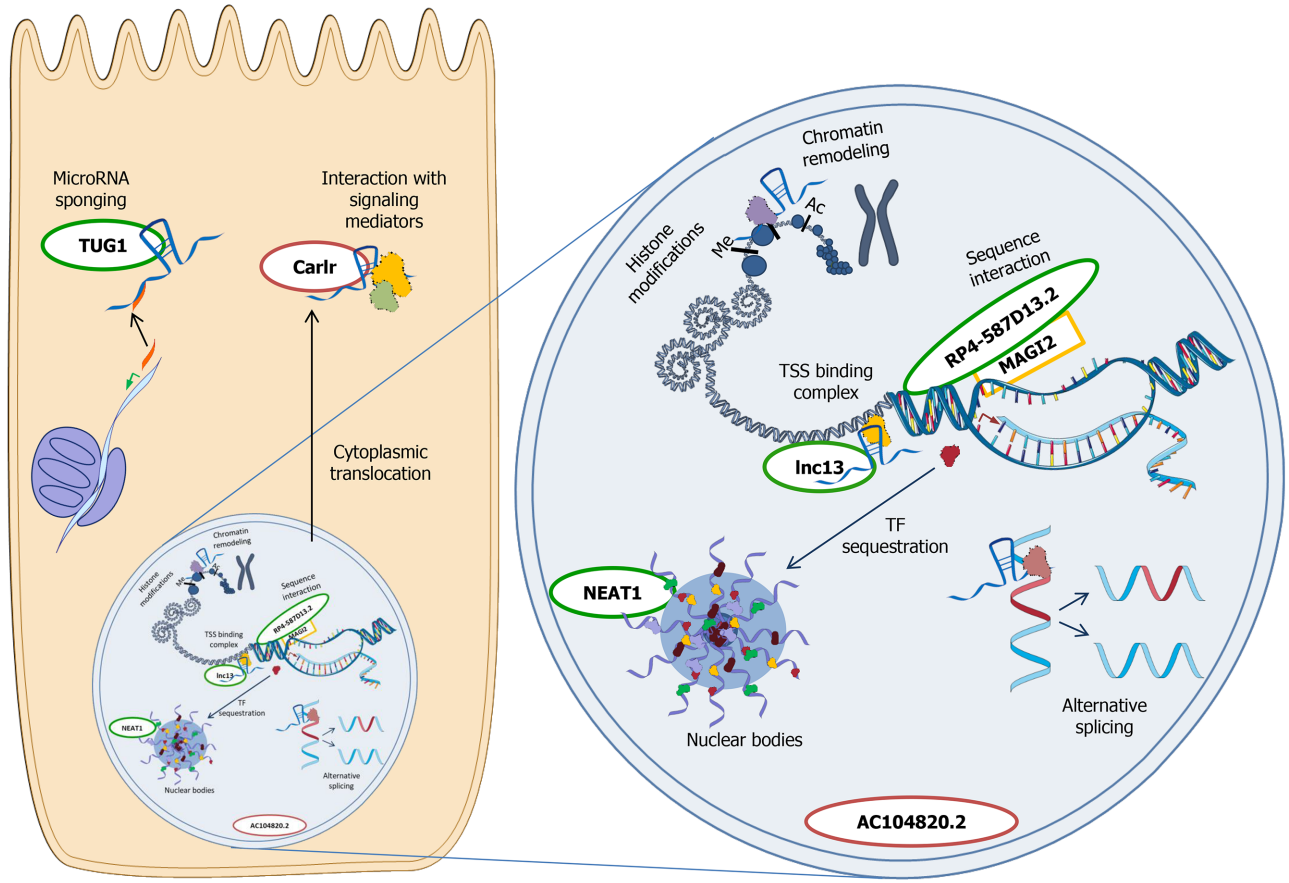


Figure 2 Main mechanisms of action of long non-coding RNAs in the cell. Cytoplasm - microRNA sponging: microRNAs are sequestered from their mRNA targets, resulting in mRNA translation; Interaction with signalling mediators: binding to pathway mediators can modulate downstream targets activation. Nucleus - chromatin remodeling: Long non-coding RNAs (lncRNAs) interact with chromatin and modify its conformation; histone modifications: lncRNAs influence the activity of the enzymes responsible for histone modifications; sequence interaction: lncRNAs act on nearby genes transcription; transcription start site (TSS) binding complex: lncRNAs bind ribonucleoproteins, interfering with gene transcription; transcription factor (TF) sequestration in nuclear bodies: TF are moved to nuclear bodies from the promoter region of target genes, influencing gene transcription. Nuclear bodies like paraspeckles consist in a lncRNA scaffold and target proteins; alternative splicing: lncRNAs determine a preferential splicing in favour of an isoform respect to another one. The so-far investigated lncRNAs in celiac disease (CeD) are reported near their known mechanism of action, respecting their main cellular localization. lncRNAs in green were reported to be downregulated in CeD, whereas the ones in red were found upregulated in a specific cell compartment. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). TSS: Transcription start site; TF: Transcription factor.

on gene transcription. Interestingly, in CeD patients, not only lnc13 expression is reduced, but there is also a SNP that generates a lnc13 variant that binds hnRNPD less effectively, possibly contributing to CeD development[50]. NFkB activation is central in CeD-driven inflammation, and it is able to induce the expression of another lncRNA, *i.e.*, Carlr. This molecule is localized in the nucleus, but it translocates in the cytosol after the activation of the NFkB pathway, and it seems essential for the induction of downstream genes such as IL-1β in macrophages. However, differently from what expected, Carlr was downregulated in total biopsies from active CeD patients, although its cytosolic fraction was increased; this observation thus needs further evaluation to determine the real role of this lncRNA in the intestinal tissue[51].

Finally, it is known that IL-15 production has a central role in CeD pathogenesis [52]. Zorro *et al*[36] in their work highlighted a subset of ncRNAs that were downregulated by IL-15 stimulation, suggesting the involvement of this class of transcripts in IL-15 response. A recent study by our group demonstrated that gliadin induces TUG1 and NEAT1 expression in biopsies obtained from CeD patients on gluten-free diet, mechanism that depends on innate immunity activation in the case of NEAT1. In fact, IL-15 can trigger NEAT1 expression both *ex vivo* and in vitro, through the newly identified axis IL-15/STAT3/NEAT1[53]. The effects of NEAT1 upregulation deserve more investigation, since this lncRNA localizes in the nucleus and is able to bind transcription factors, thus sequestering them and, in turn, regulating the expression of several genes, such as IL-8[54].

RNA METHYLATION

A further regulation can be played by RNA modifications. In fact, recent findings are pointing out that direct sequence methylation can also happen at RNA level. Differently from DNA, RNA can undergo many more kinds of methylation (up to 72), involving both mRNA and non-coding RNAs. Previous studies found RNA methylation linked to biological and immune processes, as well as complex pathologies like obesity[55]. Up to now, the main limitation in RNA methylation studies was the difficulty to perform a site-specific detection and quantification. Using a recently developed method that allows to detect m6A, the most frequent RNA modification, Olazagoitia-Garmendia *et al*[56] characterised the CeD-associated SNP rs3087898, located in the 5' untranslated region of exportin1 gene, a modulator of NFkB pathway. The mRNA derived from the CeD-associated variant was preferentially methylated with respect to the normal one, and this modification was able to increase the protein production, resulting in an inflammatory microenvironment. Besides, gliadin was able to increase this modification and exportin1 protein levels, both *in vitro* and in CeD patients samples[56]. Altogether, these studies suggest that attention should be given also to this kind of modifications when focusing on gene expression regulation in CeD.

MICRORNAS

MicroRNAs (miRNAs) are short RNA sequences (20-25 nucleotides) accounting for about 30% of gene expression regulation, affecting various processes, including chromatin conformation and transcription, as well as mRNA stability and translation. Usually they present an inverse relationship with their targets, inducing their downregulation.

miRNAs can also be detected in the plasma, and for this reason they have been evaluated in a large number of different disorders, in the attempt to identify specific biomarkers for early diagnosis or follow up. Initial papers on CeD screened the duodenal tissue to identify a specific signature representative of the events in the mucosa, and to determine whether the "signature" miRNAs were returning to normal levels in patients on GFD. Capuano *et al*[57] evaluated biopsies of children with CeD at diagnosis or on GFD, identifying about 20% of the analyzed miRNAs as differentially regulated. The authors then focused on the upregulation of miR-449a[57], which targets Notch1 and KLF4, genes involved in intestinal cell proliferation and differentiation. Indeed Notch1 expression was significantly downregulated in CeD biopsies, confirming the regulatory role of miRNAs also on proliferation in CeD. Other miRNAs were also downregulated, *i.e.*, miR-124a, miR-189, miR-299-5p and miR-379, similarly to what reported in Crohn's disease[58].

Studies on adult CeD patients identified different subset of miRNAs according to the clinical picture or the severity of intestinal damage[12,14]. A significant downregulation of miR-31-5p was observed in all the CeD groups, whereas miR-192-3p and miR-192-5p were downregulated in CeD patients with anemia and a severe histological lesion, respectively. miR-192-5p targeted two different molecules involved in the innate immunity, *i.e.*, NOD2 and CXCL2, that were upregulated in CeD patients, in particular in severe cases[12]. On the other hand, miR-31-5p had as a target Foxp3, essential for Treg development; again, a significant inverse correlation was observed between the miRNA and the target mRNA. These changes were induced by gliadin, as demonstrated by *in vitro* stimulation of biopsies of patients on GFD[12]. It is interesting to note that the data on miR-192 are similar to those observed in IBDs, in which miR-192 regulated NOD2 expression[59].

However, a more comprehensive approach was needed, and a recent paper correlated, in the duodenal mucosa of celiac subjects, the miRNA and mRNA expression patterns obtained by sequencing[17]. The data analysis revealed the presence of a complex network involving various pathways known to be deregulated in CeD, such as immunity (interferon), suggesting that CeD-associated miRNAs play a central role in causing the intestinal damage.

The identification of a specific miRNA plasma signature remains elusive at the present time. An analysis performed in pediatric CeDs revealed the presence of a trend similar to that observed in duodenal biopsies for miR-192-5p, miR-31-5p and miR-21-5p. However none of these miRNAs could be defined as a marker able to evaluate the recovery of the mucosa on GFD, since there was no return to normal levels (miR192-5p) or a clear cut-off could not be established (miR-31-5p and miR-21-5p)[13]. A

further study performed by Amr *et al*[60] analyzed the same miRNAs, *i.e.*, miR-21 and miR-31, identifying a cutoff value and determining a sensitivity and specificity of 82.4%and 80.8% for miR-21 and 93.8% and 72% for miR-31. Another recent paper confirmed the value of plasmatic miR-21 as a marker, with a sensitivity and specificity of 0.65 and 0.83, but better results were obtained considering miR-155 (0.94 and 0.87, respectively)[61]. Although encouraging, these results need further validation in larger cohorts; moreover even for miR-21 the identified cutoff values were quite dissimilar, thus suggesting the need to determine the cutoff according to the method and equipment employed for the analysis. Last but not least, the data obtained in CeD patients will have to be compared with those obtained from subjects with other inflammatory disorders such as IBDs, to confirm the specificity of the findings.

MACHINE LEARNING

As described, the evaluation of the epigenome generates large datasets, and the data increases when the integration of different datasets (such as those derived from methylome and long/short non-coding RNA transcriptome) is required. The generation of a large quantity of data allows to move towards a data-driven analysis, *i.e.*, machine learning or artificial intelligence (these terms are now regarded as interchangeable). Machine learning can, through statistical methods and algorithms, make classifications or predictions of input data, such as recognize a specific endoscopy pattern and classify the patient as celiac or non-celiac. In simpler machine learning approaches supervised learning is commonly used, *i.e.*, the use of labeled datasets designed to train or "supervise" algorithms into classifying data or predicting outcomes. In the medical field this obviously requires training datasets selected by specialized clinicians (for example the identification of endoscopic images characteristic of celiac disease). Deep learning is a subset of machine learning, in which the algorithms are more complex and include more than one layer in the neuronal network. For deep learning the presence of a labeled training dataset is not required, since these algorithms can analyze data in the raw form and determine the features that distinguish the various groups.

With regards to CeD, the machine learning approach has been originally used to analyze endoscopic data, either those generated by upper endoscopy[62] or by videocapsule and, in this latter case, the deep learning approach was able to reach a high sensitivity and specificity in the diagnosis of CeD using the video capsule images [63,64]. Although these data seem quite encouraging, it must be kept in mind that the gastrointestinal tract is a complex environment, and several factors can affect the obtained images, as discussed in depth by Hegenbart *et al*[65]. The deep learning approach could be useful also in the evaluation of duodenal biopsies, as demonstrated by different authors that were able to develop artificial intelligence-based methods for the correct classification of duodenal samples[66-68]. Machine learning has also been employed to improve the diagnosis based on B/T cell repertoire[69,70]; although the data obtained in the latter case are quite interesting, this approach is not easily applicable for routine diagnosis.

A machine learning approach could have a very important clinical application should it be able to predict who, within a risk population, will develop the disease. An encouraging study that analyzes this aspect has recently been published by Piccialli *et al*[71], who tested different models in order to predict the development of an overt CeD in childen with potential CeD. The use of a machine learning approach towards basic science data obtained from duodenal biopsies of CeD patients has recently been published by Wolf *et al*[16], who analyzed not only the trascriptomic profiles but also the expression of some lncRNAs and miRNAs. Moreover, the authors focused on gene expression signatures which associate with transcription factor (TF)-activity and chromatin state, as well as DNA and histone methylation pattern, thus providing an indirect measure of epigenetic modifications. This comprehensive analysis revealed, for example, a different role of miRNAs and lncRNAs in the regulation of gene expression, correlating the latter category with more subtle adjustments of the transcription under control of epigenetic mechanisms.

CONCLUSION

Understanding the epigenetics of celiac disease remains mandatory in order to completely clarify the pathogenetic processes behind its development and the

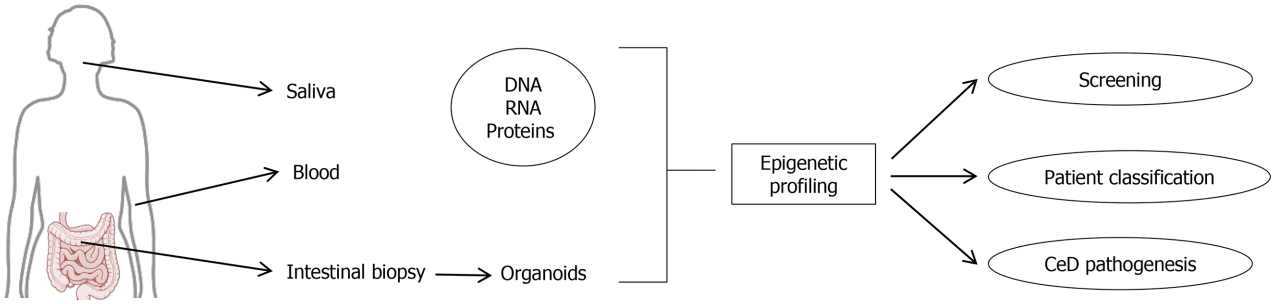


Figure 3 Potential use of epigenetics studies in celiac disease. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). CeD: Celiac disease.

spectrum of its manifestations. The data here reviewed demonstrate that epigenetic changes occur in celiac disease, however further data are still needed before the identification of these changes could be used to screen or better categorize the patients, as depicted in Figure 3. In particular it will be essential to verify that the characteristics detected at a peripheral level (*i.e.*, blood or saliva) correspond to those present in the intestinal mucosa. In this regard, data seem encouraging for miRNAs, but the identification of a specific signature will probably require the combination of more than one of them to provide an excellent sensitivity and specificity.

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ORIGINAL ARTICLE

Basic Study

Knockdown of DEAD-box 51 inhibits tumor growth of esophageal squamous cell carcinoma *via* the PI3K/AKT pathway

Dong-Xin Hu, Qi-Feng Sun, Lin Xu, Hong-Da Lu, Fan Zhang, Zhen-Miao Li, Ming-Yan Zhang

ORCID number: Dong-Xin Hu 0000-0003-0846-7361; Qi-Feng Sun 0000-0001-7498-2447; Lin Xu 0000-0002-0888-5785; Hong-Da Lu 0000-0001-5602-1191; Fan Zhang 0000-0002-9981-1609; Zhen-Miao Li 0000-0002-0905-8001; Ming-Yan Zhang 0000-0001-9995-2770.

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Dong-Xin Hu, Qi-Feng Sun, Lin Xu, Hong-Da Lu, Fan Zhang, Zhen-Miao Li, Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250000, Shandong Province, China

Ming-Yan Zhang, Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250000, Shandong Province, China

Corresponding author: Ming-Yan Zhang, MD, Associate Chief Physician, Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, No. 324, Jingwuwei Seventh Road, Huaiyin District, Jinan 250000, Shandong Province, China. zhangmingyan@sdfmu.edu.cn

Abstract

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent malignancies that seriously threaten people's health worldwide. DEAD-box helicase 51 (DDX51) is a member of the DEAD-box (DDX) RNA helicase family, and drives or inhibits tumor progression in multiple cancer types.

AIM

To determine whether DDX51 affects the biological behavior of ESCC.

METHODS

The expression of DDX51 in ESCC tumor tissues and adjacent normal tissues was detected by Immunohistochemistry (IHC) analyses and quantitative PCR (qPCR). We knocked down DDX51 in ESCC cell lines by using a small interfering RNA (siRNA) transfection. The proliferation, apoptosis, and mobility of DDX51 siRNA-transfected cells were detected. The effect of DDX51 on the phosphoinositide 3-kinase (PI3K)/AKT pathway was investigated by western blot analysis. A mouse xenograft model was established to investigate the effects of DDX51 knockdown on ESCC tumor growth.

RESULTS

DDX51 exhibited high expression in ESCC tissues compared with normal tissues and represented a poor prognosis in patients with ESCC. Knockdown of DDX51 induced inhibition of ESCC cell proliferation and promoted apoptosis. Moreover, DDX51 siRNA-expressing cells also exhibited lower migration and invasion rates. Investigations into the underlying mechanisms suggested that DDX51 knock-

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down induced inactivation of the PI3K/AKT pathway, including decreased phosphorylation levels of phosphate and tensin homolog, PI3K, AKT, and mammalian target of rapamycin. Rescue experiments demonstrated that the AKT activator insulin-like growth factor 1 could reverse the inhibitory effects of DDX51 on ESCC malignant development. Finally, we injected DDX51 siRNA-transfected TE-1 cells into an animal model, which resulted in slower tumor growth.

CONCLUSION

Our study suggests for the first time that DDX51 promotes cancer cell proliferation by regulating the PI3K/AKT pathway; thus, DDX51 might be a therapeutic target for ESCC.

Key Words: Esophageal squamous cell carcinoma; DDX51; PI3K/AKT pathway; Tumor growth; Therapeutic target

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Core Tip: Our study revealed that DEAD-box helicase 51 was upregulated in esophageal squamous cell carcinoma (ESCC) tumor tissues and promoted tumor proliferation and development by upregulating the phosphorylation of phosphoinositide 3-kinase/AKT pathway members. These data extend our knowledge of the function and molecular mechanism of the DEAD-box family in tumor biology and provide a potential therapeutic target for ESCC treatment.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the sixth leading cause of cancer-related deaths, causing approximately 300,000 deaths each year[1-3]. China has the highest incidence of ESCC globally, caused by multiple factors such as diet and the environment. At present, surgical treatment is the most effective means to cure ESCC. However, surgical treatment is highly challenging due to the close distance between the esophagus and life-supporting structures such as the respiratory system[4]. Chemotherapy and radiation therapy are commonly used as adjuvant treatments. The main problem with these two treatments is inherent resistance, and even local ESCC is often resistant[5,6]. Due to the extremely high tumor recurrence rate (about 40%) and frequent local lymph node metastases, the prognosis of ESCC patients is very poor, with a 5-year survival rate of only 10%-30%[1]. Molecular targeted therapy has shown good application prospects in ESCC patients in recent years. To improve the survival of ESCC patients, it is essential to explore effective molecular targets for ESCC treatment.

DEAD-box (DDX) RNA helicases, belonging to helicase superfamily 2, comprise the largest group of RNA helicases[7]. There are at least 31 members of DDX family in humans, including DEAD-box helicase 51 (DDX51). DDX proteins are highly conserved helicases that catalyze duplex unwinding and structural remodeling of RNA or ribonucleoprotein complexes[8]. They play important roles in nearly all aspects of RNA metabolism including RNA transcription, post-transcriptional splicing, ribosome assembly, and RNA degradation[9]. In recent years, DDX family members have been identified to be dysregulated and function as tumor oncogenes or suppressors in various tumor types[10]. For example, knockdown of DDX5 exerts inhibitory effects on the proliferation and epithelial-to-mesenchymal transition of ESCC cells by downregulating β-catenin, c-Myc, and cyclin D1[11]. DDX51 is a recently identified member of the DDX family. DDX51 is mainly responsible for maturation of the 3′ end of 28S and its proliferation-promoting activities have been

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demonstrated in non-small cell lung cancer (NSCLC) and breast cancer[12,13]. However, whether DDX51 plays a role in the progression of ESCC is still unclear.

In this study, we investigated the function of DDX51 in ESCC cell proliferation and tumor growth by using a small interfering RNA (siRNA) silencing approach.

MATERIALS AND METHODS

Tumor specimen collection and immunohistochemistry analysis

ESCC tumor tissues and adjacent normal tissues (n = 118) were obtained from patients with ESCC between 2016 to 2020 in Shandong Provincial Hospital Affiliated to Shandong First Medical University (Shandong, China). The Research Ethics Committee approved all experimental procedures of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Patients or their guardians wrote the informed consents. The clinicopathological parameters of ESCC patients were collected. Immunohistochemistry (IHC) staining was performed to investigate the expression of DDX51 in ESCC tissues and adjacent normal tissues, and the experimental procedure is described in a previous report[14].

Cell culture and transfection

The ESCC cell lines Eca109 and TE-1 were purchased from the Cell Bank of the Chinese Academy. The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin in a 37 °C cell incubator. When cells were grown to 70% confluence, si-DDX51 or negative control siRNA (si-NC) was transfected into Eca109 and TE-1 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, United States) according to the corresponding experimental groups. The stable DDX51 knockdown ESCC cell line was established by using lentivirus transfection. Lentivirus expressing si-DDX51 or si-NC was purchased from Genechem (Shanghai, China). After 48 h of transfection, cells were collected and analyzed for DDX51 expression using quantitative PCR (qPCR) and western blot analysis.

RNA extraction and qPCR

Total RNA was extracted from ESCC tumor tissues and cell lines using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, United States). Then 1 μg total RNA was reverse-transcribed to cDNA using the PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd., Dalian, China). The expression of DDX51 was determined *via* qPCR with the FastStart Universal SYBR Green Master (Roche, Germany). The experiment was performed on the C1000 Thermal Cycler (Bio-Rad, Hercules, CA, United States). The PCR reaction conditions were as follows: Pre-degeneration at 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 10s, and 72 °C for 10 s. GAPDH was used as an internal control.

Cell Counting Kit-8 assay

After transfection, Eca109 and TE-1 cells were seeded into 96-well plates at a density of 3000 cells per well. Each group had three wells. The Cell Counting Kit-8 (CCK-8; Dojindo, Kyushu, Japan) was used to detect the cell viability of transfected cells. At specific time points (24, 48, 72, and 96 h), 10 μL CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. Cell viability was represented by the absorbance at 450 nm, which was detected on a spectrophotometer (Molecular Devices, San Jose, CA, United States).

Flow cytometry

Cell apoptosis was detected by using the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, United States) following the manufacturer's instructions. The transfected cells were digested with 0.25% EDTA-free trypsin and resuspended in binding buffer. Then 100 μL cell suspension was incubated with 5 μL AnnexinV-FITC and 5 μL PI for 30 min. The incubation was performed at 37 °C in the dark. The apoptosis percentage was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, United States).

Scratch assay

A scratch assay evaluated the effect of cell transfection on its migration ability. Eca109 and TE-1 cells were seeded in a 6-well plate at a density of 1 × 10^5 cells/mL. Then cells

were transfected as described as before. After 16 h, cell transfection was stopped and cells were continuously cultured to 100% confluence. Then a single-line scratch was created on the cell monolayer using a sterile micropipette tip. Phosphate-buffered saline was used to wash the cell debris, and serum-free medium was added to the plates. Cells were photographed at 0 and 24 h under an inverted fluorescence microscope (IX-71). The width of the scratch was measured. All experiments were performed in triplicate and repeated at least three times.

Transwell invasion assay

Cell invasion was detected using a transwell assay. Transwell inserts (6.5 mm, 8 μm pore size; CoStar Group, Washington, DC, United States) were added to the 24-well transwell plates. The inserts were coated with 50 μL of 1 mg/mL Matrigel matrix (BD Biosciences). Eca109 and TE-1 cells were transfected as described above and the cell density was adjusted to 10^6 cells/mL. Then 200 μL cell suspension was added to the upper chamber of the transwell inserts, while 600 μL medium with 10% FBS was added to the lower well. Cells were cultured at 37 °C for 24 h, followed by fixation and staining with 1% crystal violet. The invasive cells were observed under a light microscope, and five random fields were selected to take pictures.

Western blot

Total proteins from transfected ESCC cells were extracted using radioimmunoprecipitation assay buffer with a 1% protease inhibitor. The protein concentration in the extraction solution was determined using the bicinchoninic acid assay. Then 20 μg total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and proteins were electrotransferred to a PVDF membrane. The membrane was blocked in 5% bovine serum albumin containing Tris-buffered saline with Tween 20 (TBST) (10 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween-20) at room temperature for 2 h, and then incubated with primary antibodies at 4 °C overnight. The primary antibodies included phosphatase and tensin homolog (PTEN) (1:1000, ab267787; Abcam, Cambridge, MA, United States), phosphoinositide 3-kinase (PI3K) (1:1000, CY5355; Abways, Shanghai, P.R. China), phosphorylated PI3K (p-PI3K) (1:1000, CY6427; Abways), AKT (1:1000, AF6261; Affinity Biosciences, Cincinnati, OH, United States), p-AKT (1:1000, AF016; Affinity Biosciences), mammalian target of rapamycin (mTOR) (1:1000, ab134903), p-mTOR (1:1000, ab137133; Abcam), and anti-GAPDH (1:5,000; ab8245; Abcam). After washing with TBST buffer four times every 5 min, the membrane was incubated with secondary antibodies (Santa Cruz, CA, United States) for 1 h at room temperature and washed with TBST another four times. Finally, proteins were visualized by chemiluminescence (Santa Cruz Biotechnology, Dallas, TX, United States).

In vivo tumorigenesis assay

Male nude mice (6-wk-old) were obtained from Shanghai SIPPR-BK Laboratory Animal Co. Ltd. (Shanghai, P.R. China). All animal experiments were approved by the Shandong Provincial Hospital Affiliated to Shandong First Medical University. The animals were maintained in specific pathogen-free conditions in accordance with the institutional animal care and use committee regulations. ESCC cells were transfected with lentivirus-NC or lentivirus-si-DDX51 for 48 h and then resuspended in a serum-free medium. The cell density was adjusted to 2×10^7/mL. Each mouse was subcutaneously injected with 5×10^6 of ESCC cells. The tumor length and width were monitored every other day and length × width^2/2 represented tumor volume. On day 28, nude mice were sacrificed by carbon dioxide asphyxiation.

Statistical analyses

All experimental data are expressed as the mean ± standard deviation from three independent experiments. The difference between the two groups was analyzed using the Student's *t*-test. The difference among multiple groups was evaluated using one-way analysis of variance. $P < 0.05$ was considered statistically significant. GraphPad Prism 5 Software (GraphPad, San Diego, CA, United States) was used for all statistical analyses. All data in this study satisfied parametric test assumptions.

RESULTS

DDX51 is highly expressed in ESCC tumor tissues and is associated with a poor prognosis in ESCC patients

DDX51 is upregulated in breast cancer. Here, we detected the expression of DDX51 in ESCC tissues, which might provide insights into its role in ESCC. As shown in Figure 1A, we performed IHC analysis of DDX51 expression in the tumor tissues and adjacent normal tissues of patients with ESCC. The results showed strong IHC staining of ESCC tissues, which was very weak in the normal controls. Meanwhile, DDX51 expression was detected by qPCR, which also showed significantly higher expression in ESCC tumor tissues than in normal adjacent tissues (Figure 1B). Furthermore, the correlation between DDX51 expression with patients' clinicopathological features was analyzed. As shown in Table 1, DDX51 expression did not correlate with patients' age, sex, tumor size, or T stage, but was associated with tumor differentiation degree, N stage and tumor-node-metastasis (TNM) stage. Survival analysis results indicated that ESCC patients with high expression of DDX51 had lower survival rates compared to those with low expression of DDX51 (Figure 1C). These results suggest that DDX51 exhibits high expression in ESCC tumor tissues and may be associated with a poor prognosis in patients with ESCC (Table 2).

Knockdown of DDX51 plays an anti-tumor role in ESCC cells

To investigate the function of DDX51 in ESCC progression, we performed a knockdown experiment in Eca109 and TE-1 cells using siRNA transfection. The transfection efficiencies are shown in Figure 2A-C. The qPCR results indicated that, compared with the NC group, the mRNA expression of DDX51 in the si-DDX51 group was decreased to 44.5% in Eca109 cells and 18.8% in TE-1 cells (Figure 2A and B, both $^{a}P < 0.05$). Western blot analysis showed that si-DDX51 transfection led to a decrease in DDX51 protein expression to 49.2% in Eca109 cells and 52.7% in TE-1 cells (Figure 2C, both $^{a}P < 0.05$), demonstrating the effective knockdown of DDX51 in both cell lines. Next, we investigated the effects of DDX51 knockdown on the malignant progression of ESCC cells. Cell viability and flow cytometry results suggested that the proliferation rates of Eca109 and TE-1 cells were significantly inhibited by DDX51 knockdown (Figure 2D and E), with a significant increase in cell apoptotic rates (Figure 3A and B). Furthermore, the wound healing rates and invasive cell number were significantly decreased in the si-DDX51 group compared with the NC group (Figure 3C-F). Taken together, these data show that DDX51 functions as an oncogene in ESCC progression.

The anti-tumor effects of DDX51 knockdown on ESCC are partly mediated by the PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway is constitutively activated in multiple cancer types and extensively participates in tumor cell proliferation, apoptosis, metastasis, and other malignant phenotypes. Here, we determined whether the oncogenic function of DDX51 in ESCC involves the PI3K/AKT signaling pathway. As shown in Figure 4A-E, the expression of members in the PI3K/AKT signaling pathway including PTEN, p-PTEN, PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR was evaluated by western blotting. The results showed that DDX51 knockdown induced a significant decrease in the phosphorylation levels of PTEN, PI3K, AKT, and mTOR, while the total protein was not affected, suggesting that the PI3K/AKT signaling pathway was inactivated in DDX51-knockdown ESCC cells. To further elucidate the role of the PI3K/AKT signaling pathway in the function of DDX51, we applied an AKT agonist insulin-like growth factor 1 (IGF-1), which induced persistent activation of the AKT pathway. IGF-1 treatment reversed the anti-proliferation and pro-apoptotic effects of DDX51 knockdown in ESCC cells (Figure 4F and G). Meanwhile, the migration and invasion abilities were also increased to the NC level when ESCC cells were treated with both si-DDX51 and IGF-1 (Figure 5). These results suggest that the anti-tumor effects of DDX51 knockdown in ESCC are, at least in part, mediated by the PI3K/AKT signaling pathway.

Knockdown of DDX51 slows down the growth of ESCC cells

We further investigated the effects of DDX51 knockdown on ESCC tumor growth *in vivo*. TE-1 cells were transfected with lv-si-DDX51 or lv-NC for 48 h and subcutaneously injected into the right flanks of nude mice. Six repeats were designed for each group. The tumor volume was measured and analyzed every other day. At the end of the experiment, tumor weights were measured and DDX51 expression was

Table 1 Correlation of DDX51 expression with the clinicopathologic features of ESCC patients

Characteristics	Cases (*n* = 118)	DDX51 expression level		*P* value
		Low (*n* = 52)	High (*n* = 66)	
Age (yr)				0.740
< 50	30	14	16	
≥ 50	88	38	50	
Gender				0.201
Male	91	43	48	
Female	27	9	18	
Tumor size				0.194
< 30 mm	20	12	8	
30-50 mm	66	29	37	
≥ 50 mm	32	11	21	
Differentiation degree				0.025[a]
High	26	11	15	
Moderate	53	30	23	
Low	39	11	28	
pT				0.154
T1	8	6	2	
T2	51	23	28	
T3 + T4	59	23	36	
pN				0.012[a]
N0	42	25	17	
N1 + N2	76	27	49	
pTNM				0.002[a]
I + II	37	24	13	
III + IV	81	28	53	

[a]*P* < 0.05 indicated statistical significance. Statistical analysis was performed using the chi-square test. TNM: Tumor-node-metastasis.

Table 2 Comparison of Kaplan–Meier survival curves

	B	SE	Wald	*P* value	HR	95%CI
Differentiation	0.052	0.157	0.110	0.740	1.053	0.774-1.433
pN	0.029	0.451	0.004	0.949	1.029	0.425-2.491
pTNM	1.078	0.518	4.323	0.038	2.939	1.064-8.119
DDX51	0.893	0.241	13.686	< 0.01	2.443	1.522-3.921

analyzed by IHC analysis. The results are shown in Figure 6, which indicated that the tumor volume of the lv-si-DDX51 group (1639.35 ± 674.36 mm^3) was significantly decreased compared with that of the NC group (1013.03 ± 264.51 mm^3) on day 28. The weight of the NC group (0.442 ± 0.116 g) was also significantly higher than that of the lv-si-DDX51 group (0.715 ± 0.156 g). These results showed that DDX51 knockdown inhibited ESCC tumor growth in the mouse xenograft model.

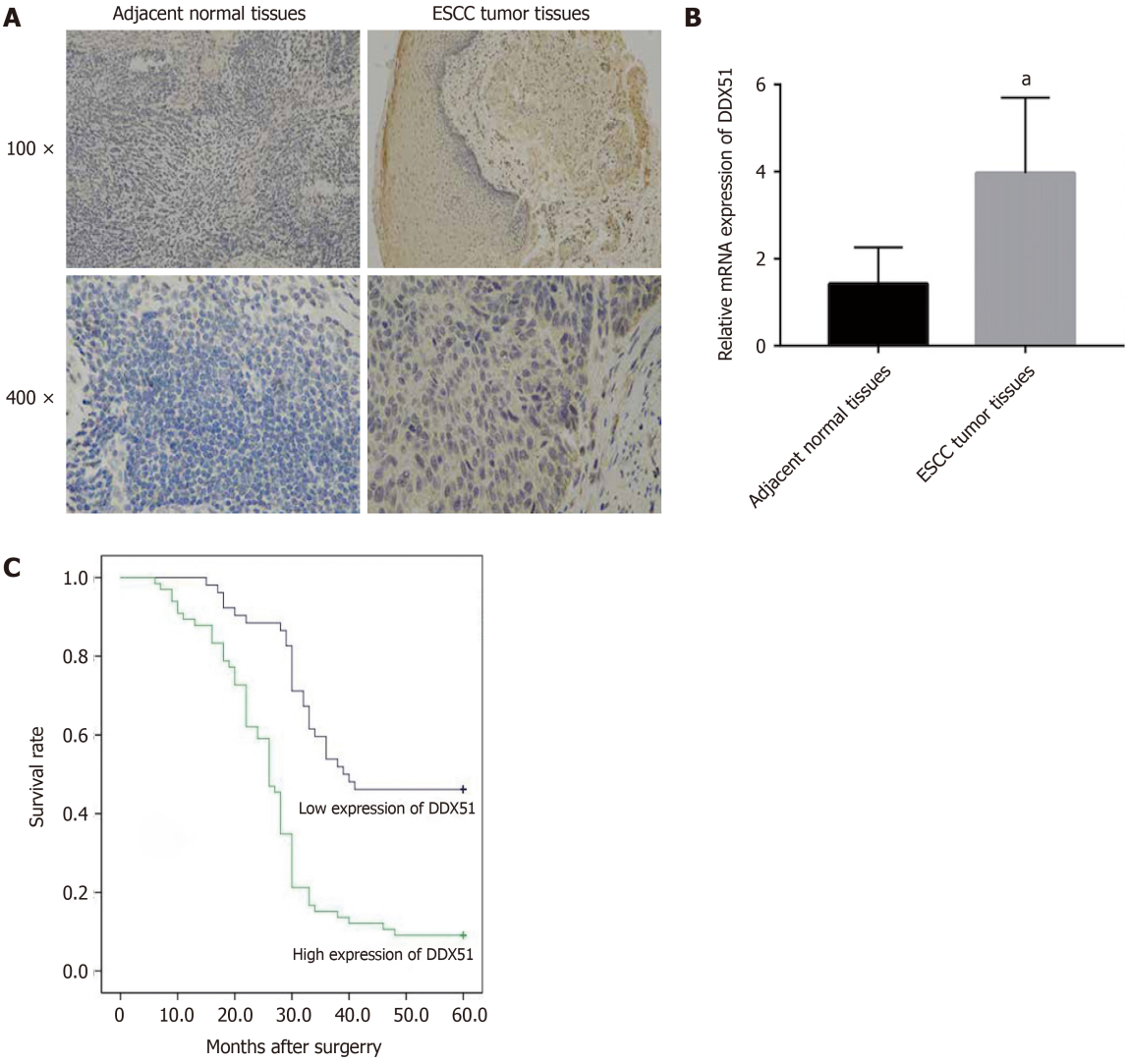


Figure 1 Expression and prognostic value of DEAD-box helicase 51 in esophageal cancer. A: Immunohistochemistry analysis of DEAD-box helicase 51 (DDX51) expression in tumor tissues and paired normal tissues of patients with esophageal squamous cell carcinoma (ESCC); B: Quantitative PCR detection of DDX51 mRNA expression in the tumor tissues (n = 118) and paired normal tissues (n = 118) of patients with ESCC, $^{a}P < 0.05$; C: Survival curves of ESCC patients with high or low expression (P = 0.0036).

DISCUSSION

The DDX family, which has activities of RNA unwinding and structure remodeling, regulates nearly all aspects of RNA-related biology, from synthesis to degradation [10]. Since mRNA serves as an intermediate between DNA genetic information and proteins, the DDX family plays crucial roles in multiple cellular processes such as cell proliferation, apoptosis, and malignant transformation[15-19]. DDX proteins ordinarily exert their function by forming a large multi-protein complex. Therefore, their exact function is probably impacted by their interacting partners and is profoundly context dependent[20,21]. The most important characteristic of a tumor is heterogeneity, which might explain the diverse functions of DDX proteins in tumors, as an oncogene or tumor suppressor[22,23]. DDX51 is a recently identified member of DDX family, which shows pro-proliferation activity in the progression of NSCLC and breast cancer[12,13].

Here we demonstrated that DDX51 was upregulated in ESCC tumor tissues and promoted tumor proliferation and metastasis. The results also suggested that the oncogenic function of DDX51 in ESCC was a result of activation of the PI3K/AKT signaling pathway, which is consistent with previous reports that revealed the regulatory action of other DDX proteins on the AKT pathway.

DDX family members are dysregulated in various tumor types, including ESCC [10]. For example, gene microarray data of breast cancer showed that DDX1 upregulation is associated with tumor occurrence and early recurrence and serves as an

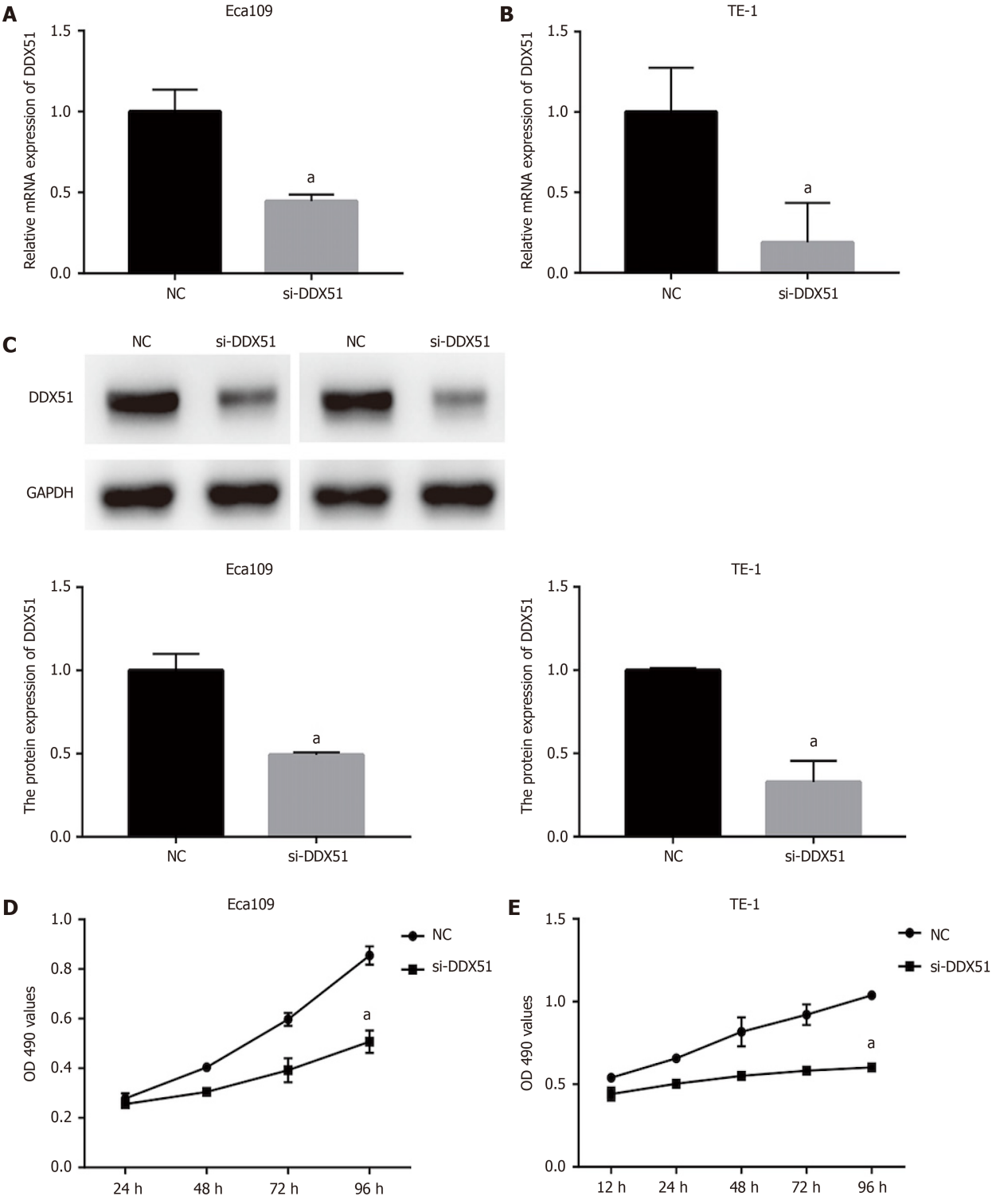
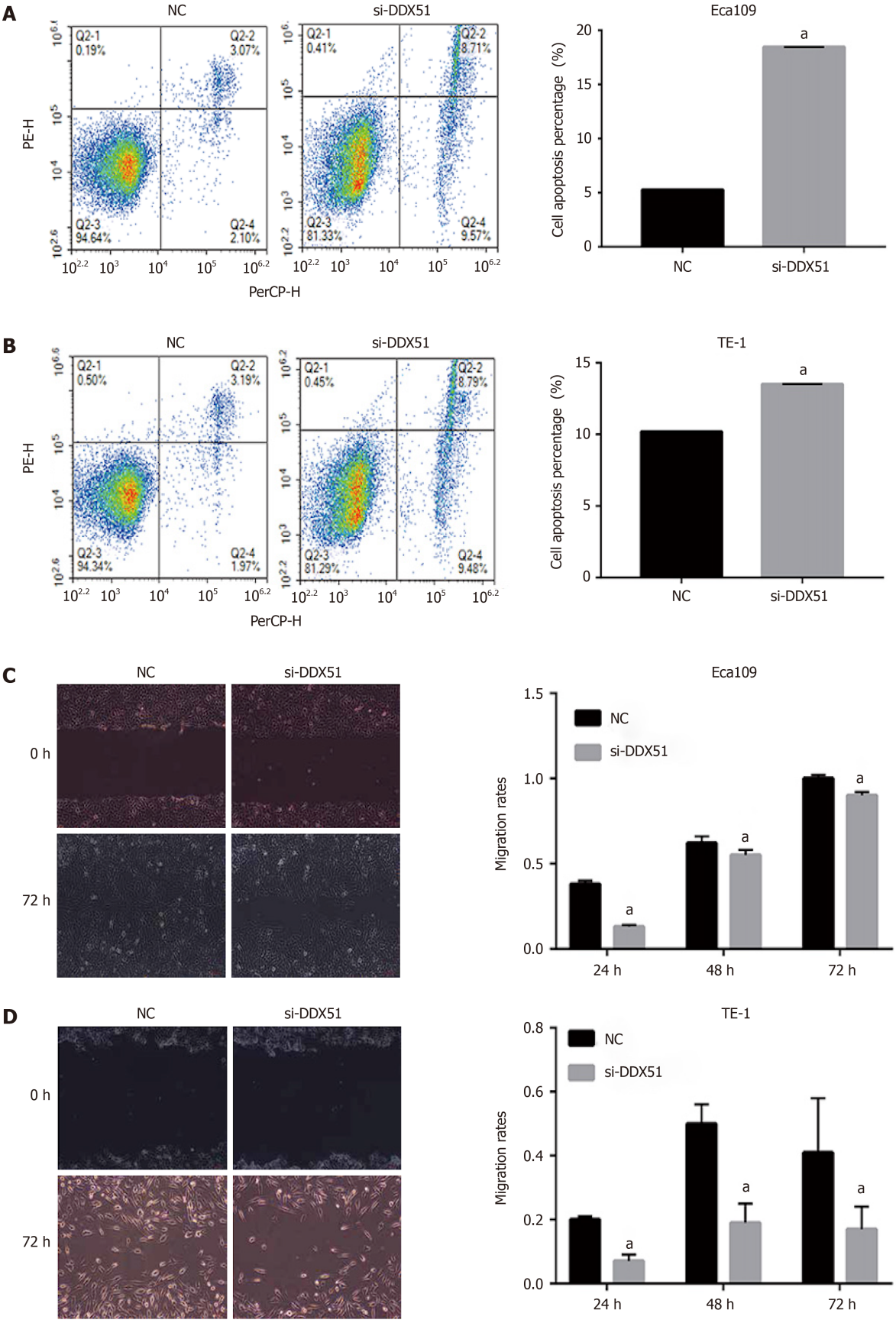


Figure 2 Anti-proliferation effects of DEAD-box helicase 51 in esophageal cancer. Negative control (NC) small interfering RNA (siRNA) and DEAD-box helicase 51 (DDX51) siRNA were synthesized and transfected into Eca109 and TE-1 cells. A and B: Quantitative PCR assay was used for the detection of knockdown efficiencies; C: Western blot analysis of DDX51 expression in DDX51 siRNA-expressing cells; D and E: The effects of DDX51 on the viability of esophageal squamous cell carcinoma cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. NC was a scrambled siRNA.

independent prognostic biomarker for patients' survival[24]. Wang *et al*[12] investigated the expression of DDX51 in NSCLC patients. The results showed that DDX51 expression was associated with patient age but no other risk factors. In breast cancer, DDX51 also exhibits high expression in tumor tissues and predicts a high TNM stage and poor prognosis in patients[13]. Here, our results showed that DDX51 was upregulated in ESCC tumor tissues and was negatively correlated with patient survival, which was consistent with previous studies on DDX51 in tumors. Our study identified that the upregulated expression of DDX51 in ESCC is associated with the


A
NC
si-DDX51
Q2-1 0.19%
Q2-2 3.07%
Q2-3 94.64%
Q2-4 2.10%
Q2-1 0.41%
Q2-2 8.71%
Q2-3 81.33%
Q2-4 9.57%
PE-H
PerCP-H
Eca109
Cell apoptosis percentage (%)
a
NC
si-DDX51
B
NC
si-DDX51
Q2-1 0.50%
Q2-2 3.19%
Q2-3 94.34%
Q2-4 1.97%
Q2-1 0.45%
Q2-2 8.79%
Q2-3 81.29%
Q2-4 9.48%
PE-H
PerCP-H
TE-1
Cell apoptosis percentage (%)
a
NC
si-DDX51
C
NC
si-DDX51
0 h
72 h
Eca109
Migration rates
NC
si-DDX51
a
24 h
48 h
72 h
D
NC
si-DDX51
0 h
72 h
TE-1
Migration rates
NC
si-DDX51
a
24 h
48 h
72 h

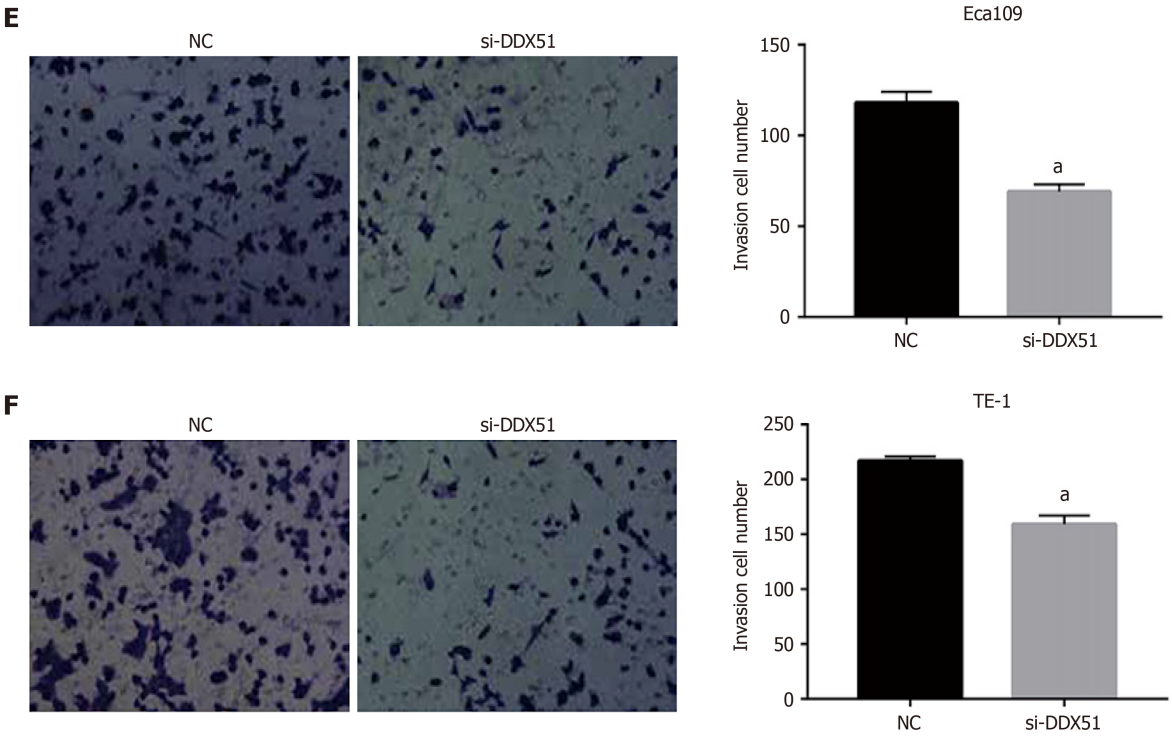


Figure 3 Effects of DEAD-box helicase 51 small interfering RNA on cell apoptosis, migration, and invasion of esophageal squamous cell carcinoma cells. A and B: Cell apoptosis percentage of DEAD-box helicase 51 (DDX51) small interfering RNA (siRNA)-expressing esophageal squamous cell carcinoma (ESCC) cells; C and D: Scratch assays for cell migration analysis of the DDX51 siRNA-expressing ESCC cells; E and F: Transwell assays for cell invasion analysis of DDX51 siRNA-expressing ESCC cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled siRNA.

degree of differentiation, pathological N stage, and pathological TNM stage. The above findings suggest that DDX51 overexpression may serve as an independent prognostic factor in ESCC. However, it is currently unclear if this is an indication of the severity of the cancer itself. Further study should be performed to investigate the prognostic value of DDX51 in a larger number of specimens.

As RNA synthesis and decay involve the regulation of broad-spectrum protein expression, it is not surprising that the dysregulation of DDX proteins in tumors affects different signaling pathways. In a review reported in 2021, the signaling regulation network of DDX family in tumorigenesis was summarized including Wnt/β-catenin pathway, Snail/E-cadherin pathway, hypoxia inducible factor 1 alpha/DDX3/E-cadherin and so on[10]. For DDX51, microarray analyses revealed that DDX51 siRNA-expressing cells expressed higher levels of transforming growth factor beta receptor, interleukin 1 receptor, and c-FOS in NSCLC[12]. The Wnt/β-catenin pathway is also downregulated by DDX51 siRNA in breast cancer[13]. Here, we found that DDX51 siRNA resulted in the inactivation of the PI3K/AKT pathway, including decreased phosphorylation levels of PTEN, PI3K, AKT, and mTOR. The PI3K/AKT pathway is constitutively activated in many tumor types including ESCC, and participates in tumor cell proliferation, apoptosis, and progression[25-27]. In a previous study, DDX5 was demonstrated to occupy the AKT promoter with β-catenin as well as nuclear factor kappa B, and induces the mRNA and protein expression of AKT[28]. However, in gastric cancer miR-5590-3p targets DDX5, which further decreases the phosphorylation levels of AKT and does not affect AKT protein expression[29]. These results suggest that DDX proteins can regulate the AKT pathway through either protein expression or phosphorylation level. In the future, we intend to investigate the effects of DDX51 on transcriptome and proteome expression in tumor progression, which will provide more information on the mechanism of action of this protein.

CONCLUSION

This study had some limitations. First, *in vivo* experiments were not performed to

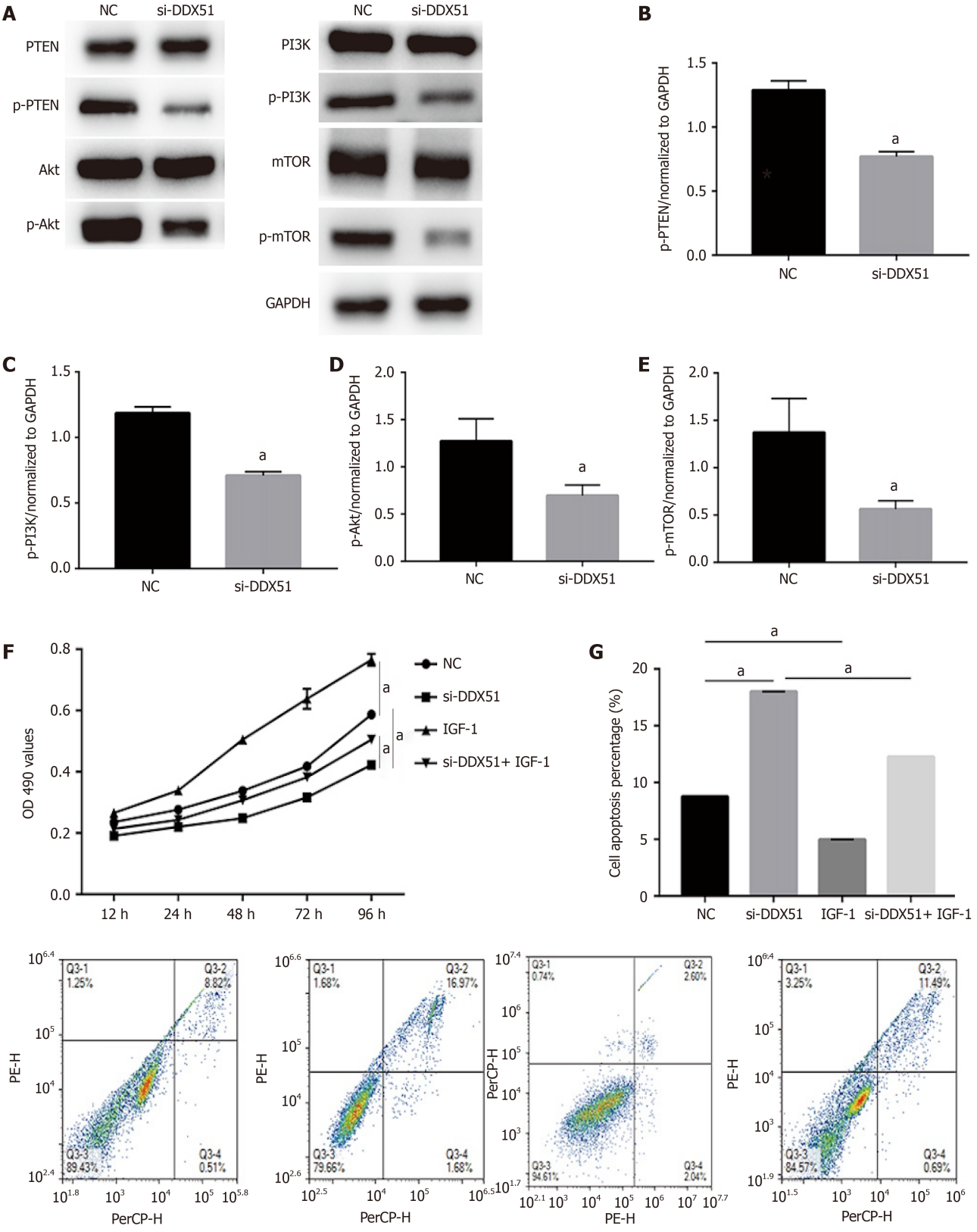


Figure 4 Knockdown of DEAD-box helicase 51 induced inactivation of the phosphoinositide 3-kinase/AKT signaling pathway. A-E: Expression of phosphoinositide 3-kinase (PI3K)/AKT pathway members, including phosphatase and tenin homolog (PTEN), phosphorylated PTEN (p-PTEN), PI3K, p-PI3K, AKT, p-AKT, mammalian target of rapamycin (mTOR) and p-mTOR, was detected by western blot analysis; F: AKT activator insulin-like growth factor 1 (IGF-1) reversed the inhibitory effect of DDX51 knockdown on the proliferation of TE-1 cells; G: AKT activator IGF-1 reversed the pro-apoptotic effect of DDX51 knockdown on the proliferation of TE-1 cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled small interfering RNA.

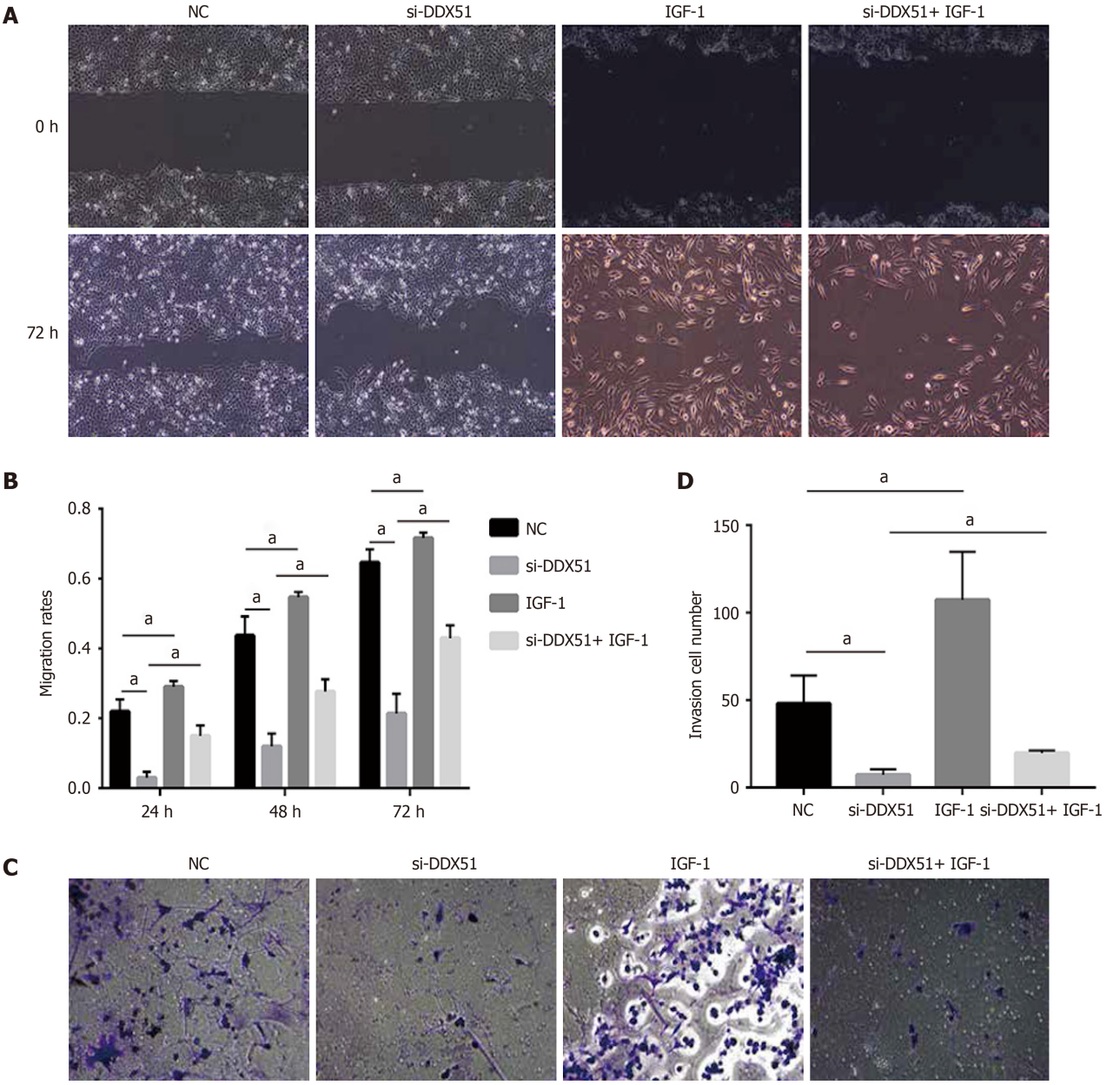


Figure 5 Anti-migration and anti-invasion effects of DEAD-box helicase 51 knockdown was mediated by the AKT pathway. A and B: AKT activator insulin-like growth factor 1 (IGF-1) reversed the anti-migration effect of DDX51 knockdown on the proliferation of TE-1 cells; C and D: AKT activator IGF-1 reversed the anti-invasion effect of DDX51 knockdown on the proliferation of TE-1 cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled small interfering RNA.

further confirm the effect of DDX51 knockdown on lung and lymph node metastases. Second, the exact regulatory mechanism of DDX51 in ESCC was not explored. Future studies are required to further confirm the potential of DDX51 as a therapeutic target for the targeted treatment of ESCC.

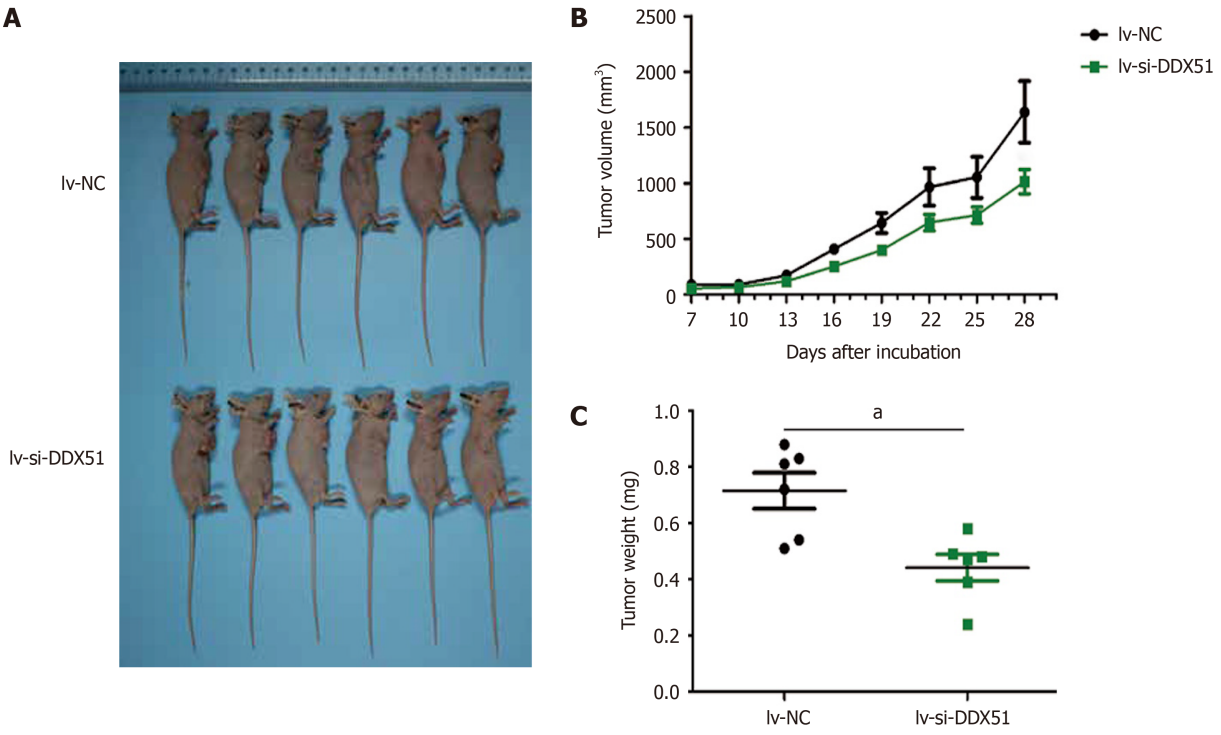


Figure 6 DEAD-Box helicase 51 knockdown inhibited the tumor growth of esophageal squamous cell carcinoma *in vivo*. TE-1 cells transfected with lentivirus-small interfering-negative control (lv-si-NC) (n = 6) or lv-si-DEAD-box helicase 51 (DDX51) (n = 6) were injected into nude mice and tumor volumes were monitored. A: The photographs of mice with esophageal squamous cell carcinoma tumor of TE-1 cells transfected with lv-si-NC or lv-si-DDX51; B: Tumor growth curves; C: At the end of experiments, mice were executed, tumors were excised and tumor weights were measured. $^{a}P < 0.05$. Negative control was a scrambled siRNA.

ARTICLE HIGHLIGHTS

Research background

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent malignancies that seriously threatens people's health worldwide. DDX51 is a member of the DEAD-box (DDX) RNA helicase family, which drives or inhibits tumor progression in multiple cancer types.

Research motivation

To identify the role of DDX51 in ESCC and the molecular mechanisms involved.

Research objectives

To explore the effect of DDX51 on ESCC progression.

Research methods

The expression of DDX51 in ESCC tumor tissues and adjacent normal tissues was detected by immunohistochemistry analysis and quantitative PCR (qPCR). We knocked down DDX51 in ESCC cell lines using small interfering RNA (siRNA) transfection. The proliferation, apoptosis, and mobility of DDX51 siRNA-transfected cells were detected. The effects of DDX51 on the phosphoinositide 3-kinase (PI3K)/AKT pathway were investigated using western blot analysis. A mouse xenograft model was established to investigate the effects of DDX51 knockdown on ESCC tumor growth.

Research results

DDX51 exhibited high expression in ESCC tissues compared with normal tissues and was associated with a poor prognosis in patients with ESCC. Knockdown of DDX51 induced inhibition of ESCC cell proliferation and promoted apoptosis. Moreover, DDX51 siRNA-expressing cells also exhibited lower migration and invasion rates. Investigation into the mechanism of action suggested that DDX51 knockdown induced inactivation of the PI3K/AKT pathway including decreased phosphorylation levels of PTEN, PI3K, AKT and mTOR. Rescue experiments demonstrated that the AKT

activator insulin-like growth factor 1 could reverse the inhibitory effects of DDX51 on ESCC malignant development. Finally, we injected DDX51 siRNA transfected TE-1 cell into an animal model, which resulted in slower tumor growth.

Research conclusions

Our study suggests for the first time that DDX51 contributes to ESCC cell proliferation by regulating the PI3K/AKT signaling pathway.

Research perspectives

DDX51 may serve as a potential therapeutic target for the treatment of ESCC.

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ORIGINAL ARTICLE

Basic Study

Fibrinogen-like protein 2 deficiency inhibits virus-induced fulminant hepatitis through abrogating inflammatory macrophage activation

Fang Xiao, Hong-Wu Wang, Jun-Jian Hu, Ran Tao, Xin-Xin Weng, Peng Wang, Di Wu, Xiao-Jing Wang, Wei-Ming Yan, Dong Xi, Xiao-Ping Luo, Xiao-Yang Wan, Qin Ning

ORCID number: Fang Xiao 0000-0002-7153-454X; Hongwu Wang 0000-0002-4351-3371; Junjian Hu 0000-0002-7043-1638; Ran Tao 0000-0002-8912-9861; Xinxin Weng 0000-0002-9902-1783; Peng Wang 0000-0002-6962-8012; Di Wu 0000-0012-7083-826X; Xiaojing Wang 0000-0002-9042-7253; Weiming Yan 0000-0002-7942-4034; Dong Xi 0000-0002-5013-2649; Xiaoping Luo 0000-0002-2942-4834; Xiao-Yang Wan 0000-0002-8871-2015; Qin Ning 0000-0002-2027-9593.

Author contributions: Wan XY and Xiao F wrote the manuscript; Wan XY, Ning Q and Wang HW designed projects, interpreted data and developed concept of the project; Xiao F, Hu JJ and Weng XX did the experiments; Tao R and Wang P collected samples; Xi D, Wan XY, Wang XJ and Luo XP participated the conceptual discussion of the paper; Ning Q and Wang HW revised the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Tongji Hospital, Wuhan Province.

Institutional animal care and use committee statement: All animal experiments were approved by

Fang Xiao, Hong-Wu Wang, Jun-Jian Hu, Ran Tao, Xin-Xin Weng, Peng Wang, Di Wu, Xiao-Jing Wang, Wei-Ming Yan, Dong Xi, Xiao-Yang Wan, Qin Ning, Department of Infectious Disease, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Fang Xiao, Department of Infectious Disease, The First Affiliated Hospital of Guangxi Medical University, Nanning 530000, Guangxi Province, China

Xiao-Ping Luo, Department of Pediatrics, Tongji Hospital, Wuhan 430030, Hubei Province, China

Corresponding author: Qin Ning, Doctor, PhD, Chief Doctor, Professor, Research Scientist, Department of Infectious Disease, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jiefang Avenue, Wuhan 430030, Hubei Province, China. qning@vip.sina.com

Abstract

BACKGROUND

Heterogeneous macrophages play an important role in multiple liver diseases, including viral fulminant hepatitis (VFH). Fibrinogen-like protein 2 (FGL2) is expressed on macrophages and regulates VFH pathogenesis; however, the underlying mechanism remains unclear.

AIM

To explore how FGL2 regulates macrophage function and subsequent liver injury during VFH.

METHODS

Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH in FGL2-deficient (*Fgl2-/-*) and wild-type (WT) mice. The dynamic constitution of hepatic macrophages was examined. Adoptive transfer of *Fgl2-/-* or WT bone marrow-derived macrophages (BMDMs) into WT recipients with macrophages depleted prior to infection was carried out and the consequent degree of liver damage was compared. The signaling cascades that may be regulated by FGL2 were detected in macrophages.

RESULTS

institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008), and conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

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Following MHV-3 infection, hepatic macrophages were largely replenished by proinflammatory monocyte-derived macrophages (MoMFs), which expressed high levels of FGL2. In *Fgl2-/-* mice, the number of infiltrating inflammatory MoMFs was reduced compared with that in WT mice after viral infection. Macrophage depletion ameliorated liver damage in WT mice and further alleviated liver damage in *Fgl2-/-* mice. Adoptive transfer of *Fgl2-/-* BMDMs into macrophage-removed recipients significantly reduced the degree of liver damage. Inhibition of monocyte infiltration also significantly ameliorated liver damage. Functionally, *Fgl2* deletion impaired macrophage phagocytosis and the antigen presentation potential and attenuated the proinflammatory phenotype. At the molecular level, FGL2 deficiency impaired IRF3, IRF7, and p38 phosphorylation, along with NF-κB activation in BMDMs in response to viral infection.

CONCLUSION

Infiltrated MoMFs represent a major source of hepatic inflammation during VFH progression, and FGL2 expression on MoMFs maintains the proinflammatory phenotype *via* p38-dependent positive feedback, contributing to VFH pathogenesis.

Key Words: Viral fulminant hepatitis; Fibrinogen-like protein 2; Proinflammatory macrophages; Infiltrating macrophages; P38

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Core Tip: In this study, we demonstrate that: (1) Monocytes infiltrating the liver represent a major source of hepatic inflammation, which has a decisive effect on the pathogenesis of viral fulminant hepatitis; (2) Viral infection induces FGL2 expression on macrophages, which is required for maintaining the inflammatory phenotype and cell function; and (3) FGL2 generates a positive feedback loop of an inflammatory cascade in macrophages in response to viral infection.

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INTRODUCTION

Viral fulminant hepatitis (VFH) is a type of acute liver failure (ALF) that can develop after viral infection in patients with hepatitis A, hepatitis B, and hepatitis E. VFH is a devastating syndrome characterized by severe liver injury with coagulation abnormalities and hepatic encephalopathy[1]. Pathologically, acute liver injury and subsequent massive hepatocyte loss are caused by hyperactivation of the innate immune response, followed by an excessive adaptive immune response to viral infection, leading to systemic inflammation. Innate immune cells, activated by pathogen-associated molecular patterns (PAMPs) from invading pathogens, damage-associated molecular patterns (DAMPs) released from necrotic cells, and afferent endotoxins from the portal vein, are dominant in conditions of sterile inflammation and non-virus-associated ALF[2,3]. Adaptive immunity activated by PAMPs seems to play a more important role in VFH than innate immunity because of the exposure to abundant viral antigens presented by antigen-presenting cells[4,5]. However, the extent of the contribution of innate immunity in generating viral antigens or DAMPs in VFH is not well defined.

Liver-resident macrophages, also known as Kupffer cells (KCs), are the largest population of hepatic immune cells that play an essential role in maintaining liver homeostasis and ensure rapid responses to hepatic insults[6]. Monocyte-derived macrophages (MoMFs) are an ontologically distinct subpopulation of hepatic

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macrophages, which are found in only small numbers under physiological conditions and then serve to replenish the macrophage population during liver injury[7]. Macrophages are generally classified into proinflammatory (M1) and anti-inflammatory (M2) macrophages (or alternatively activated macrophages), phenotypically corresponding to T helper (Th)1 and Th2 responses, respectively. However, the spectrum of macrophage classification remains obscure as the transition between macrophage subtypes occurs in response to microenvironment mediators[8,9]. Dynamic alterations in KC and MoMF populations were observed in the context of sterile inflammation in an experimental model of ALF induced by acetaminophen overdose, with replacement of KCs by MoMFs during disease progression[10]. Further analyses of this model revealed differential subsets of macrophages showing the protective and detrimental characteristics of KCs and MoMFs during the course of acetaminophen-induced ALF[10,11]. Moreover, an increase in the number of MoMFs with potent activity has been observed in non-pregnant patients with hepatitis E-associated ALF compared with that in pregnant patients. In a model of experimental VFH, hepatic leukocyte numbers were found to increase at an early stage of liver injury[12], suggesting the involvement of macrophages in disease progression. However, further investigation is needed to elucidate the precise roles of the distinct subpopulations of hepatic macrophages in VFH.

Fibrinogen-like protein 2 (FGL2), a membrane protein with prothrombinase at the N-terminal, is expressed in a variety of cells, such as macrophages, dendritic cells, and endothelial cells, and can be induced robustly and exclusively in macrophages in response to stimulation with cytokines [interferon (IFN)-gamma or tumor necrosis factor (TNF)-α), viral infection, and lipopolysaccharide (LPS)[7,13]. This suggests that FGL2 itself is a critical mediator of inflammation in that the interaction between inflammation and coagulation is reciprocally exacerbated in terms of inflammatory cytokine production and tissue factor secretion[11]. FGL2 deficiency was shown to prevent the development of VFH in mice following experimental murine hepatitis virus strain 3 (MHV-3) infection; this effect was speculated to be mainly dependent on its ability to regulate procoagulant activity, as evidenced by concomitant expression of FGL2 and fibrinogen production[14]. Emerging evidence suggests that FGL2 is a downstream effector molecule in the inflammatory cascades associated with VFH development and progression, which involve C5aR, TNF-α, and MSR1[7,12,15,16]. Interestingly, complement activation, a critical step for inflammation initiation, has also been observed in FGL2-deficient mice following MHV-3 infection[16], highlighting a complex regulatory network of inflammation involving FGL2 and other effectors. Therefore, we hypothesized that FGL2 plays a role in the reconstitution of hepatic macrophages to regulate proinflammatory macrophage activation that contributes to VFH progression and exacerbation. To test this hypothesis, we first explored the hepatic macrophage composition and expression pattern of FGL2 during the progression of VFH in patients with and without hepatitis B virus (HBV)-associated acute-on-chronic liver failure (ACLF), since the pathology of ACLF closely resembles that of ALF[17]. To explore the role of FGL2 in VFH progression and the underlying mechanism, we determined alterations in hepatic macrophage populations during progression of VFH in FGL2-deficient (*Fgl2*-/-) and wild-type (WT) mice intraperitoneally injected with MHV-3 at different stages after infection.

MATERIALS AND METHODS

Ethics statement

All studies on human subjects were approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (permit number: TJ-C20170924), and all participants provided informed consent in compliance with the Helsinki Declaration revised in 1983.

The animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008) and were conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

Patient samples

Seven patients with ACLF, as defined by the Asian Pacific Association for Study of the Liver (APASL) guidelines[18], who were undergoing liver transplantation were included in the study; liver samples were obtained during the surgery. The clinical features of the patients are summarized in Table 1. Control samples (n = 3) were

Table 1 Clinical characteristics of patients and controls

Characteristics	ACLF (*n* = 7)	Healthy controls (*n* = 3)
Age (year)	47.14 ± 3.3[a]	30.33 ± 3.4
Sex (male:female)	6:1	3:0
Laboratory evaluation		
ALT (U/L)	642.9 ± 360.3	8.67 ± 1.76
Tbil (μmol/L)	429.8 ± 70.53[a]	17 ± 2.34
PTA (%)	30.29 ± 3.3[a]	118 ± 12.17
HBsAg (ng/mL)	3870 ± 1600	0
HBV-DNA (10^6 copies/mL)	8.18 ± 6.78	0
Ascites and/or Encephalopathy	7	0

[a]$P < 0.5$ *vs* controls.

Age, Serum alanine aminotransferase, total bilirubin, prothrombin time activity shown as mean ± SD. ACLF: Acute on chronic liver failure; ALT: Alanine aminotransferase; Tbil: Total bilirubin; PTA: Prothrombin time activity.

obtained from a healthy liver donor and from the paratumor tissues of a patient with hepatic hemangioma and a patient with hepatocellular carcinoma without cirrhosis.

Animals, viral infection, macrophage depletion, and inhibitor treatment

Female BALB/c mice (HFK Bioscience Company Ltd., Beijing, China) at 6-8 weeks of age were used in all animal experiments. The mice were housed in a controlled environment (specific pathogen-free, 12 h light/dark cycle, 21 ± 2 °C, humidity 50 ± 10%) and had free access to food and water. *Fgl2-/-* mice were generated by introducing a deletion at the N-terminal of the open reading frame of the *Fgl2* locus using CRISPR/Cas9 technology. WT BALB/c mice were used as controls. To establish the fulminant hepatitis model, 100 plaque-forming unit (PFUs) of MHV-3 (American Type Culture Collection, Manassas, VA, USA) dissolved in 200 μL saline was injected into the WT and *Fgl2-/-* mice intraperitoneally. To deplete macrophages, mice were intravenously injected with 200 μL clodronate liposomes or phosphate-buffered saline (PBS)-liposomes as a control (Liposome B.V., the Netherlands) 24 h prior to MHV-3 injection. To inhibit MoMF infiltration, a chemokine receptor-2 (CCR2) inhibitor (cenicriviroc; 10 mg/kg) was intraperitoneally injected 24 h prior to MHV-3 infection. WT or *Fgl2-/-* mice were randomly selected to be sacrificed at 0, 24, 48, and 72 h following MHV-3 injection, and liver and blood samples were harvested for analysis.

Cell isolation

Hepatic mononuclear non-parenchymal cells from WT and *Fgl2-/-* mice were isolated *via* liver perfusion, enzymatic digestion, and differential centrifugation, as described previously[19]. Peritoneal exudate macrophages (PEMs) were isolated from the mice 3 d after intraperitoneal injection of 3% starch broth (1 mL/mice). Bone marrow-derived macrophages (BMDMs) were differentiated from hematopoietic stem cells isolated from the femurs cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and macrophage-colony stimulating factor (30 μg/mL, Peprotech) for 7 days. BMDMs or PEMs were maintained in DMEM supplemented with 10% FBS and treated with LPS (100 ng/mL), interleukin (IL)-4 (20 ng/mL), or MHV-3 (5×10^5 PFU/mL).

Macrophage adoptive transfer

KCs are the largest population of hepatic resident non-parenchymal cells and the first line of defense against pathogens and toxins[6]. The priming of KCs may therefore be involved in initiation of the hepatic immune response, a critical step for subsequent exacerbation of inflammatory accumulation. Accordingly, we also assessed liver damage following MHV-3 infection with depletion of macrophages by clodronate liposomes. WT mice were treated with 200 μL clodronate liposomes or PBS-liposomes as a control to deplete macrophages, which then served as the recipient mice for adoptive transfer. Harvested BMDMs from WT and *Fgl2-/-* mice were intravenously injected into the recipient mice at 5×10^5 cells/mouse. MHV-3 (100 PFU/mouse)

injection was performed 24 h later. Liver and blood samples were harvested at 48 h post MHV-3 infection for further analyses.

Flow cytometry

Single-cell suspensions were blocked with 2 μg/mL anti-CD16/CD32, followed by staining with fluorescence-conjugated antibody cocktails. For intracellular staining, surface-stained cells were fixed and permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Kit (No. 554714, BD Pharmingen) prior to incubation with target antibodies. Data were acquired on a BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FACS Diva software.

Detailed procedures and other materials are described in the supporting information.

Statistical analyses

Statistical testing was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). Comparisons between WT and *Fgl2*$^{-/-}$ mice (or other situations where only two groups are compared) were performed using Student's unpaired t-test; one-way analysis of variance with Tukey's *post-hoc* test or Mann-Whitney *U* test was used for multiple comparisons (unless otherwise indicated). Data are presented as mean ± standard deviation; $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

The number of proinflammatory macrophages with elevated FGL2 expression increases in patients with hepatitis virus-induced liver failure

Marked infiltration of leukocytes was observed in samples of the patients with ACLF, along with massive necrosis in the parenchymal lobules (Figure 1A). CD68+ macrophages markedly accumulated in the periportal areas (Figure 1B, Supplementary Figure 1), suggesting widespread liver damage[20]. Furthermore, macrophages were polarized to proinflammatory macrophages, as confirmed by the expression of S100A9 (Figure 1C), an alarmin molecule expressed in proinflammatory macrophages, activated neutrophils, and granulocytes, which is often used as a biomarker of inflammation[21]. Interestingly, the majority of S100A-positive activated proinflammatory macrophages also expressed FGL2 in consecutive sections of liver samples from patients with HBV-related liver failure (Figure 1D). These data suggest that a high expression level of FGL2 could be an important molecular event of hepatic macrophage polarization during viral liver failure.

Ly6C^{high} MoMFs dominate the hepatic macrophage population during VFH progression

In the experimental fulminant hepatitis mouse model, pathogenesis was divided into an early stage without global liver damage at 24 h post-infection of MHV-3, a progression phase with continuous enlargement of block necrosis and increased transaminase levels, and an end stage defined at 72 h post-infection when the animals were near death (Figure 2A and B). At the early stage of infection, hematoxylin and eosin staining showed that the hepatic architecture was intact and no obvious macrophages infiltration was noted (Figure 2A). Aggregating plots of the CD11b F4/80-expressing flow cytometry panel[22] showed a continuous increase in the number of hepatic MoMFs (CD11b^{high}F4/80^{int}) and a decline in the number of KCs (CD11b^{low} F4/80^{high}) over the course of infection, accompanied by enlarged lesions until confluent necrosis of the liver lobules occurred at the later stage (Figure 2A and C). Notably, KCs constituted the dominant hepatic macrophage population at the early stage of infection, with an increased M1 macrophage subset [induced nitric oxide synthase (iNOS+) KCs] (Figure 2D, Supplementary Figure 2A and C). However, MoMFs subsequently dominated the hepatic macrophage population, characterized by an increased ratio of Ly6C^{high} cells; however, the Ly6C^{high}/Ly6C^{low} ratio decreased at the end stage of the disease (Figure 2E, Supplementary Figure 2A), implying a transition from Ly6C^{high} MoMFs to Ly6C^{low} MoMFs. The dynamic alterations in hepatic macrophage populations were also revealed by an increase in the levels of proinflammatory cytokines TNF-α and IL-6 during progression, which then decreased at the end stage, along with a continued decrease in transforming growth factor (TGF)-β levels in macrophages (Supplementary Figure 2D). Consistent with the reconstitution of hepatic

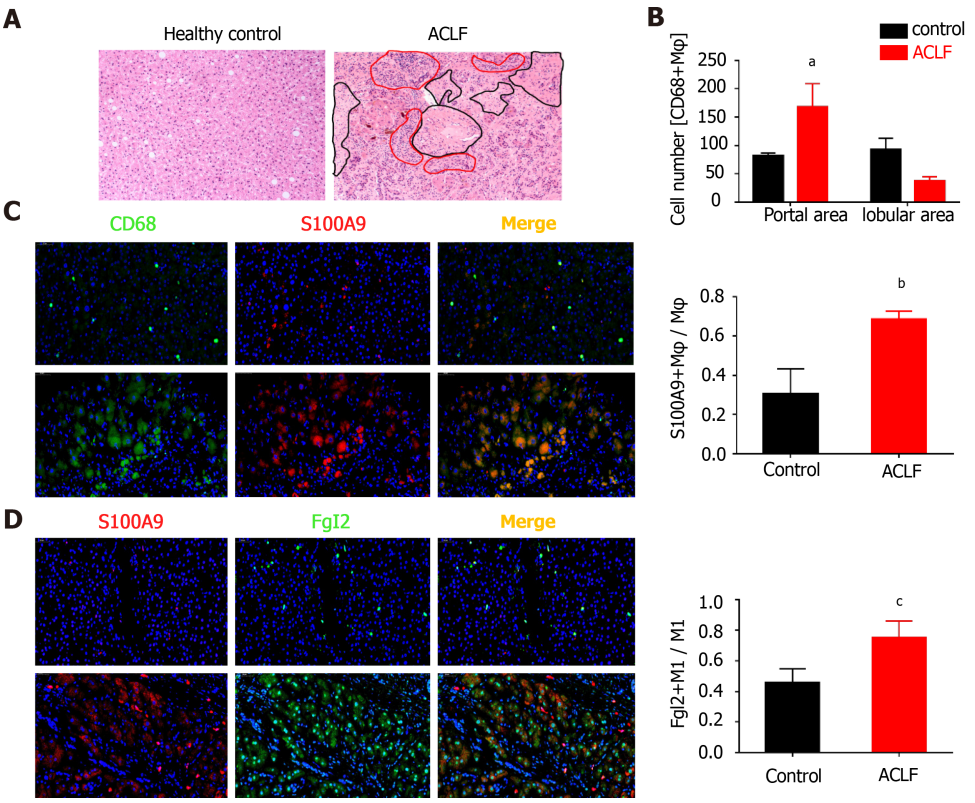


Figure 1 Fibrinogen-like protein 2 was expressed on proinflammatory macrophages in patients with hepatitis B virus-associated liver failure. A: Representative image of liver sections (200×) subjected to Hematoxylin and eosin staining (H&E); Black circles point to transparent necrosis, red circles point to infiltrating leukocytes; B: Calculation of CD68+ macrophages at periportal and lobular areas; C: Immunofluorescence against CD68, S100A9 on liver sections (200×) from patients of hepatitis B virus-associated acute chronic-on liver failure (acute-on-chronic liver failure (ACLF), *n* = 7) and healthy controls (*n* = 3); D: Immunofluorescence against S100A9 and fibrinogen-like protein 2 on liver sections (200×) from ACLF patients (*n* = 7) and healthy controls (*n* = 3), representative images on the left and statistical columns on the right. Data are expressed as mean ± SD.

macrophage populations in which Ly6C^{high} MoMFs are more inflammatory with an expanded spectrum of cytokine and chemokine expression compared with M1 macrophages[23], the serum MCP-1 level was remarkably increased during infection-induced liver failure progression (Supplementary Figure 2B).

Potent induction of FGL2 is associated with the proinflammatory phenotype of both KCs and MoMFs in response to viral infection

As shown in Figure 3A, over 90% of FGL2-positive KCs were found in iNOS+ macrophages based on flow cytometry. Moreover, Ly6C^{high} MoMFs constituted the major population of FGL2-positive MoMFs, and the expression level of FGL2 was remarkably higher in Ly6C^{high} MoMFs than in Ly6C^{low} MoMFs (Figure 3B). After viral infection, sequential induction of FGL2 expression was observed in both polarized M1 KCs and Ly6C^{high} MoMFs during disease progression, although a slight decrease in the expression level was observed when the KC and MoMF populations were somewhat exhausted at 48 and 72 h post viral infection, respectively (Figure 3C and D). However, FGL2 expression was maintained at low levels in Ly6C^{low} populations (Figure 3D). Together, these data suggested that FGL2 is markedly induced in proinflammatory macrophages upon viral infection.

FGL2 deficiency prevents proinflammatory macrophage activation after MHV-3 infection

In *Fgl2-/-* mice (Supplementary Figure 3A), liver damage following MHV-3 infection was significantly attenuated, as revealed by narrowed necrosis foci and lower alanine transaminase (ALT) and aspartate transaminase (AST) levels compared with those in WT mice (Figure 4A and B). In addition, viral titers were reduced in *Flg2*$^{-/-}$ mice compared with those in their WT counterparts (Supplementary Figure 3B). Consistent with the pathology results, monocyte infiltration was significantly reduced in *Flg2*$^{-/-}$ mice, whereas a large number of KCs was maintained during VFH progression (Figure 4C). To determine whether *Fgl2* was required for proinflammatory

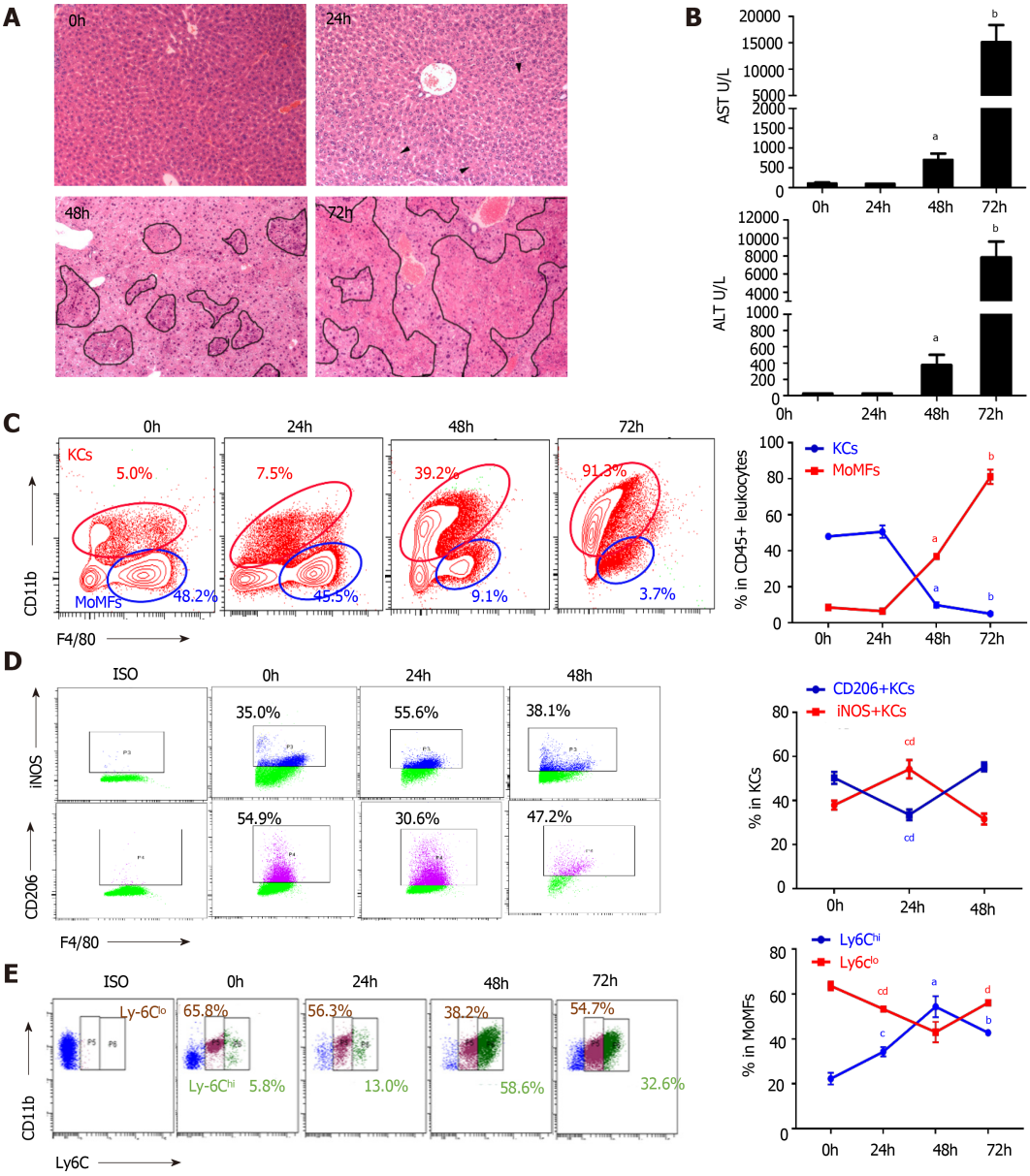


Figure 2 Dynamic alteration of macrophage subsets during viral fulminant hepatitis progression. A: Time-course H&E staining on liver sections of mice after murine hepatitis virus strain 3 infection. Arrowheads indicated hepatocyte cytoplasmic destruction, Circles pointed to necrosis; B: ALT and AST levels from serum of mice post-viral infection; C: Flow cytometry of KCs (CD45+ F4/80high), MoMFs (CD45+ Ly6C+ F4/80int) of cells (left), and their percentage in the liver at various time point after viral infection; D: Flow cytometry of KCs (left) and frequency of M1 (iNOS+), M2 (CD206+) macrophages in KCs (right); E: Representative image of MoMFs (left) and frequency of Ly6Chigh and Ly6Clow MoMFs at different time point after viral infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times. KCs: Kuffer cells; MoMFs: Monocyte derived macrophages.

macrophage activation, we investigated the polarization of KCs at the early stage of VFH when KC populations were reserved. We found a decrease in the M1 KC subset and an increase in the M2 KC numbers in FGL2-deficient mice before and after MHV-3 infection (Figure 4D), suggesting that FGL2 depletion impaired inflammatory macrophage activation and favored the M2 phenotype. As expected, a small Ly6C^{high} subset was also observed in the MoMF population in FGL2-deficient mice after viral infection, implying that *Fgl2* loss predisposed MoMFs to the Ly6C^{low} phenotype (Figure 4E). This interpretation was further supported by the reduced levels of TNF-α and IL-6 and the increased levels of TGF-β on KCs from *Fgl2*-/- mice after viral infection compared with those in WT mice (Supplementary Figure 3C).

FGL2 induces KC polarization for initiation of inflammatory events after viral infection

Macrophage depletion by clodronate liposomes significantly relieved liver injury, as shown by decreased necrosis foci and ALT/AST levels, in both WT and FGL2-deficient

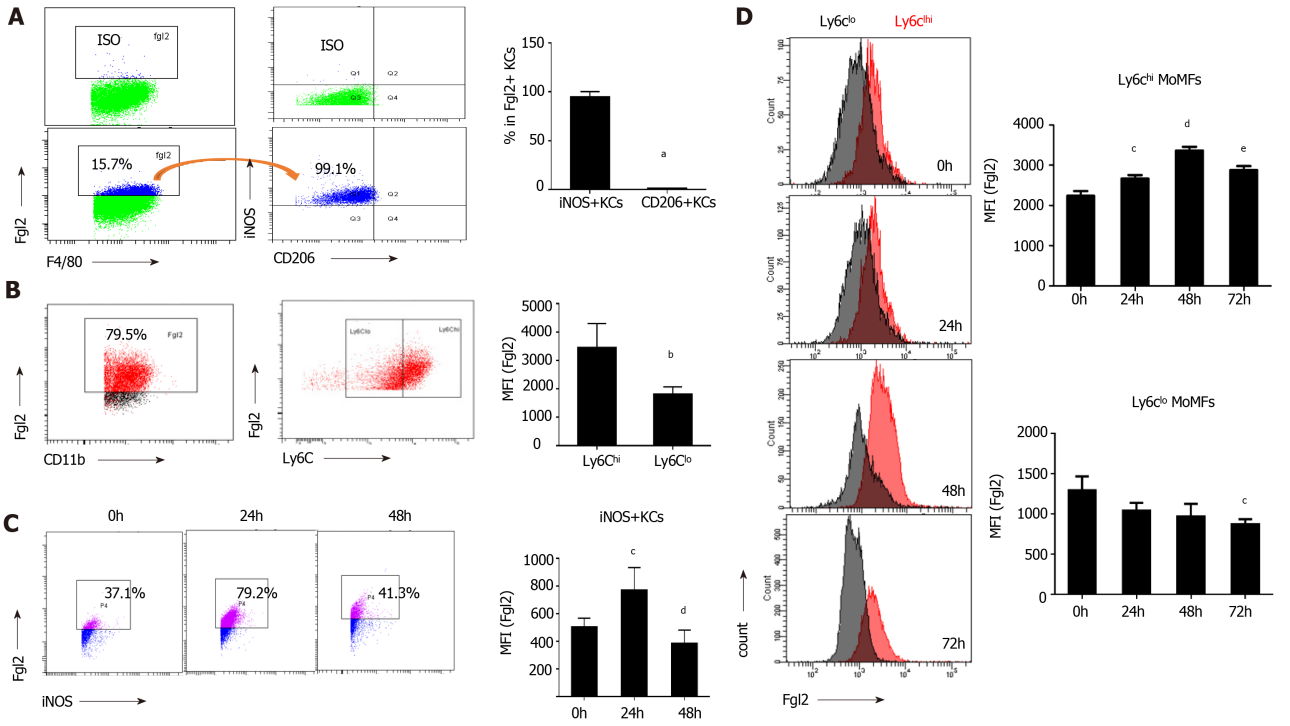


Figure 3 Fibrinogen-like protein 2 expression was robustly inducted upon proinflammatory macrophage activation. A: Flow cytometry of fibrinogen-like protein 2 (Fgl2) +KCs expressing iNOS and CD206 under homeostatic conditions (left), and frequency of iNOS+ and CD206+ cells subsets (right); B: Representative image of Fgl2+MoMFs expressing Ly6C (left), and relative expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clo MoMFs under physiological conditions (right); C: Time course-presentation of Fgl2 expression in M1 polarized KCs (left), and expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clow MoMFs, respectively (right); D: Cell counts of Fgl2+ MoMFs (left), and relative level of Fgl2 expression on both MoMFs subset (right) following viral infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times. MFI: Mean fluorescence intensity.

mice (Figure 5A and B). Notably, further reduction in liver ALT/AST levels and fewer necrotic hepatocytes were observed in FGL2-deficient mice after viral infection, suggesting the requirement of FGL2 for macrophage polarization and disease progression. To determine whether FGL2 regulates macrophage polarization directly, BMDMs from WT and *Fgl2*-/- mice were transferred into WT recipient mice with self-BMDMs depleted in advance in the VFH model. Interestingly, *Fgl2*-/- BMDMs exhibited reduced numbers of inflammatory polarized macrophages and increased numbers of anti-inflammatory macrophages (Figure 5D and E). Moreover, recipient mice adoptively transferred with *Fgl2*-/- BMDMs exhibited reduced liver injury, as revealed by histological analysis (Figure 5A) and ALT/AST levels (Figure 5C), when compared with that in recipients transferred with WT BMDMs.

As depicted in Supplementary Figure 3D and E, smaller numbers of MPO^{high} myeloid cells, which are considered to be neutrophils, were observed in liver sections from mice with macrophage depletion or in FGL2-deficient mice compared with those from untreated WT mice following MHV-3 infection. However, no significant difference in neutrophil abundance was observed between WT and *Fgl2*-/- mice with macrophage depletion.

Because MoMFs formed the largest component of myeloid cells during the acute stage and CCR2 is mainly expressed in proinflammatory monocytes (Ly6C^{high})[24], we speculated that MoMF-derived inflammation is a major source of hepatic inflammation. Indeed, treatment with the CCR2 inhibitor cenicriviroc significantly reduced the virus-induced liver damage (Figure 5F and G). Collectively, these data suggest that FGL2 directly regulates proinflammatory macrophage polarization and that infiltrated MoMFs are the major source of hepatic inflammation during acute liver injury.

FGL2 deficiency attenuates macrophage M1 polarization in vitro

To further determine whether FGL2 regulates macrophage polarization directly *in vitro*, we examined its regulatory effect on BMDMs and PEMs by LPS stimulation or MHV-3 infection. As expected, in WT BMDMs, robust production of cytokines and chemokines, such as IL-1β, TNF-α, IL-6, IL-12, and MCP-1, and reactive species, such as the NO-derived nitrite product (NO_2^-), were observed in response to either LPS treatment or MHV-3 infection. In contrast, the levels of such products were markedly

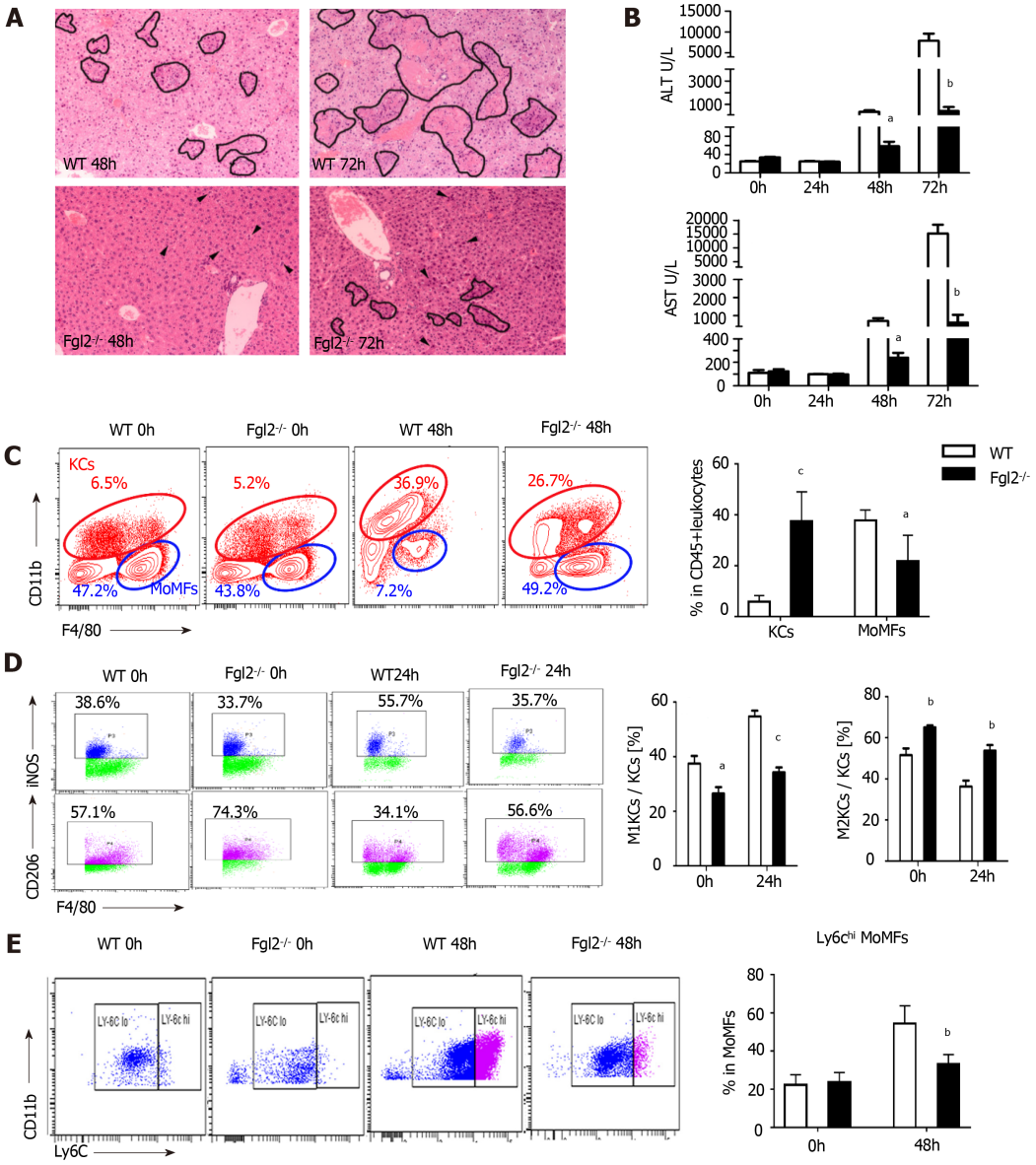


Figure 4 Fibrinogen-like protein 2 promotes pro-inflammatory macrophage polarization following murine hepatitis virus strain 3 infection.
A: H&E staining on liver sections from wild type (WT) and fibrinogen-like protein 2 (*Fgl2-/-*) mice at 48 and 72 h post viral infection. Circled field represented bulk necrosis. Arrows point to necrotic cells; B: Serum alanine transaminase and aspartate transaminase levels at 0, 24, 48, and 72 h post viral infection in WT and *Fgl2-/-* mice; C: Flow cytometry of KCs and MoMFs in hepatic CD45+ leukocytes at steady condition and 48 h following viral fulminant hepatitis in WT and *Fgl2-/-* mice (left); and respective frequency at 48 h post infection; D: Frequency of polarized M1 (iNOS+), M2 (CD206+) KCs at 0 and 24 h post murine hepatitis virus strain 3 (MHV-3) infection; E: Frequency of Ly6Chi and Ly6Clow MoMFs at 0 and 48 h post MHV-3 infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times.

reduced in BMDMs from *Fgl2-/-* mice under the same condition (Figure 6A). Consistently, the transcription levels of M1 indicators, such as NOS2, TNF-α, IL-6, IL-1 β, IL-12, and Marco, were significantly reduced in BMDMs of *Fgl2-/-* mice compared with those in BMDMs from WT mice in response to either LPS stimulation or MHV-3 infection (Figure 6B and C). In contrast, both IL-10 production and the transcriptional levels of M2 markers were significantly higher in *Fgl2-/-* BMDMs than in WT BMDMs in response to IL-4 treatment (Figure 6D and E). Similar results were also obtained in *Fgl2-/-* PEMs in response to LPS treatment (Supplementary Figure 4). Taken together, these data suggest that FGL2 deficiency impairs the proinflammatory polarization of macrophages and promotes an alternative activated phenotype in response to IL-4 stimulation.

FGL2 deficiency reduces the antigen presentation and phagocytosis of macrophages

A critical function of hepatic macrophages during homeostasis maintenance is to engulf pathogens, endotoxins, and debris of dead cells *via* a process called

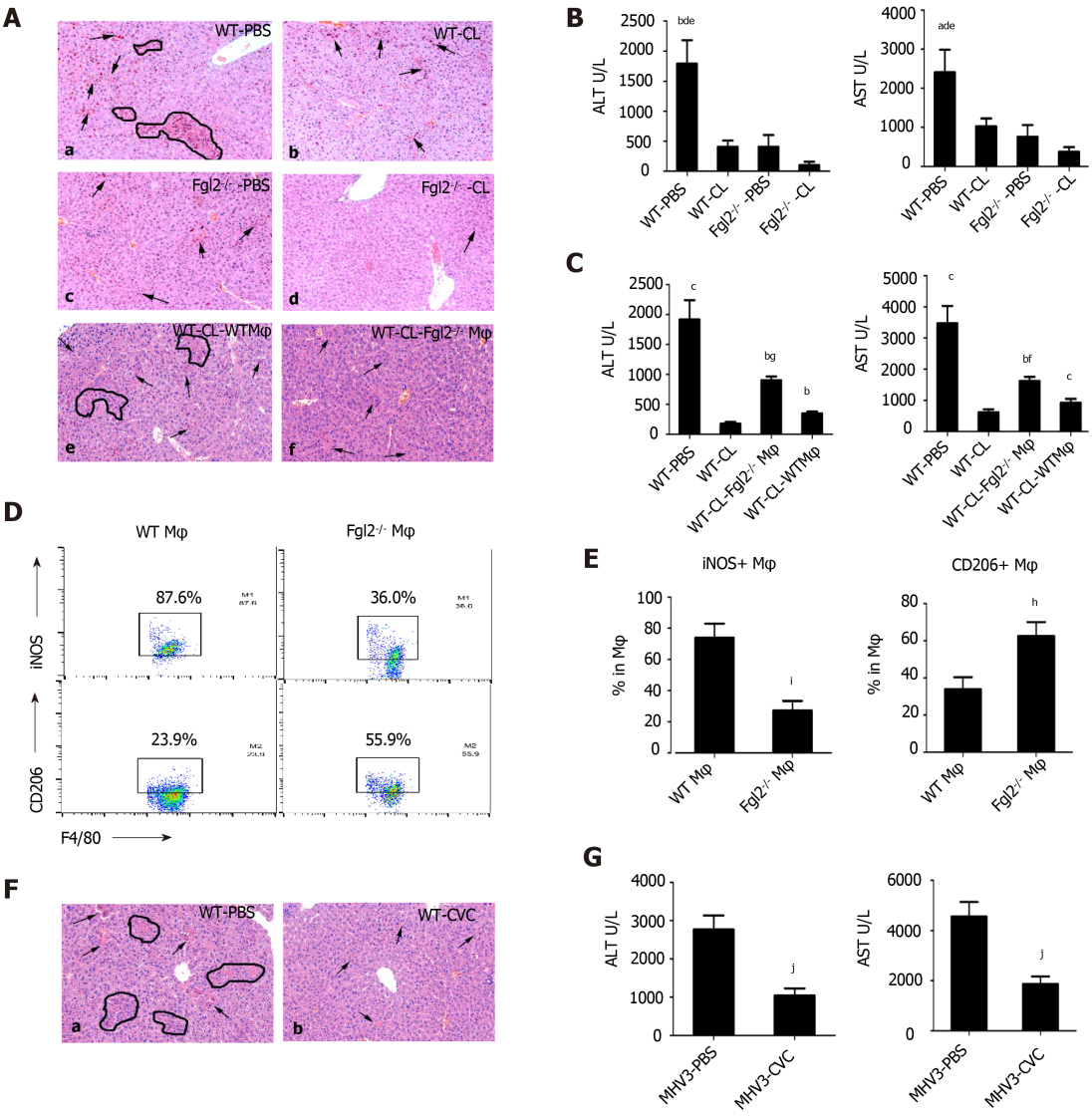


Figure 5 Depletion of macrophages in fibrinogen-like protein 2 synergically attenuated liver damage after viral infection. A: H&E staining on liver sections from mice administered with either clodronate liposomes (CL) (b and d on the right) or PBS-liposomes (PBS) (a and c on the left) at 48 h post murine hepatitis virus strain 3 (MHV-3) infection; e-f, liver sections from bone marrow-derived macrophages (BMDMs) adoptive transferred mice at 48 h post MHV-3 infection (e: WT BMDM donor; f: Fibrinogen-like protein 2 (*Fgl2*-/-) BMDM donor); B: Serum ALT and AST levels from clodronate liposomes-treated WT and *Fgl2*-/- mice at 48 h post MHV-3 infection; C: Serum AST and ALT levels from WT and *Fgl2*-/- BMDM chimeric mice; D: representative F4/80+ iNOS+ and F4/80+ CD206+ donor macrophages from WT and *Fgl2*-/- BMDM chimeric mice at 48 h post infection; E: statistical analysis of iNOS+ and CD206+ donor macrophages in chimeric mice at 48 h post infection; F: H&E-stained liver section from mice which were treated with Cenicriviroc (CVC) in advance and subjected to MHV-3 infection for 48 h. Arrows represented necrotic cells, circles represent areas of hepatocyte necrosis. Image magnificence: 200×; G: aminotransferase levels of CVC treated viral fulminant hepatitis mice and its control. Data were presented as mean ± SD (n = 5). These experiments were repeated at least three times. BMDM: Bone marrow derived macrophages.

phagocytosis, followed by antigen presentation by major histocompatibility complex (MHC) II for T cells. We therefore cultured *E. coli* labeled with red fluorescence together with peritoneal macrophages to examine their phagocytic capabilities. The number of engulfed bacteria was notably smaller in macrophages from FGL2-deficient mice than in macrophages from WT mice, although more macrophages were detected in *Fgl2*-/- mice (Figure 7A and B). Furthermore, surface expression of MHC II was more severely impaired in FGL2-deficient macrophages than in WT macrophages under normal conditions or after viral stimulation (Figure 7C), whereas expression of CD80, CD86, or MHC I was not altered (data not shown). These data suggest that FGL2 deficiency attenuates both the phagocytic capacity and antigen presentation potential of macrophages.

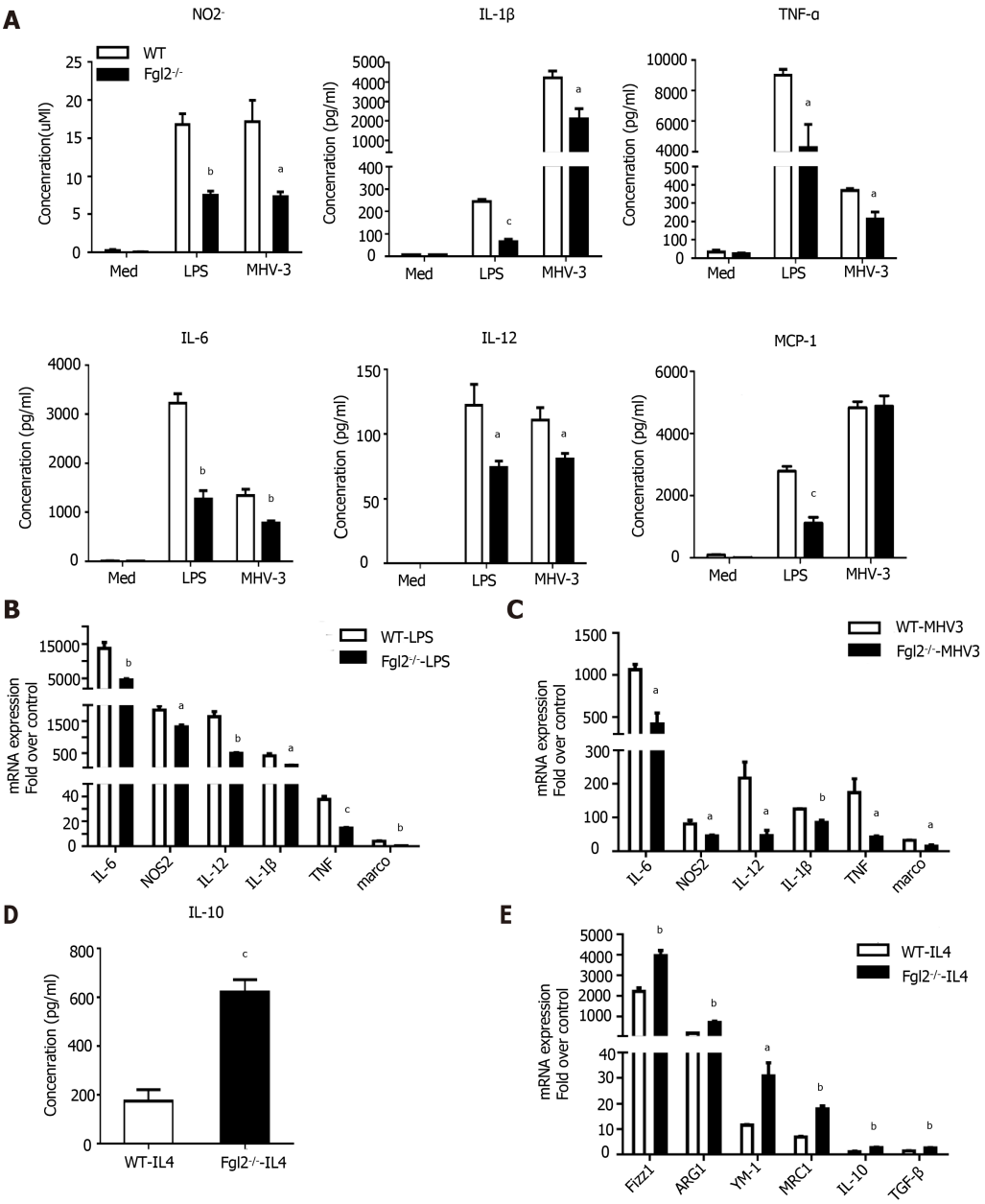


Figure 6 Fibrinogen-like protein 2 promoted bone marrow-derived macrophages M1 polarization *in vitro*. A: Supernatant concentration of NO2-, IL-1β, TNF-α, IL -6, IL-12 and MCP-1 released by bone marrow-derived macrophages (BMDMs) after LPS (100 ng/mL) treatment or murine hepatitis virus strain 3 (MHV-3) infection for 24 h; B: Relative transcriptional level of M1 markers (NOS2, IL-6, IL-12, IL-1β, TNF-α and marco) of WT and fibrinogen-like protein 2 (*Fgl2-/-*) BMDMs after the stimulation of LPS for 6 hours; C: Relative transcriptional level of M1 markers of WT and *Fgl2-/-* BMDMs after MHV-3 infection for 8 hours; D: Supernatant IL-10 concentration of BMDMs culture after the IL-4 stimulation (20 ng/mL) for 72 h; E: mRNA expression of M2 markers (Fizz1, ARG1, YM-1, MRC1, IL-10 and TGF-β) of WT and Fgl2 -/- BMDMs after of IL-4 treatment for 72 h. All data are presented as mean ± SD (n > 3). These experiments were repeated at least three times.

FGL2 modulates the inflammatory signaling cascade by activating NF-κB, IRF3, IRF7, and p38

To explore how FGL2 modulates inflammatory cascades, we examined the serial components of inflammatory signaling pathways in BMDMs by mimicking invading pathogen infections *in vitro*. Consistent with the results from MoMFs in the VFH model, FGL2 was robustly induced in BMDMs following LPS treatment and MHV-3 infection (Figure 8A). In addition, phosphorylation of IκBα, p65, IRF3, and IRF7, which are critical modulators of NF-κB activation and IFN expression, was remarkably impaired in *Fgl2-/-* BMDMs in response to LPS treatment and viral infection (Figure 8B, Supplementary Figure 5). However, the expression of adaptor proteins such as TRIF and MyD88 was not altered under the same conditions (Figure 8B). Among mitogen-activated protein kinases (MAPKs), the level of phosphorylated p38 was reduced, whereas the levels of activated (phosphorylated) JNK and ERK were not

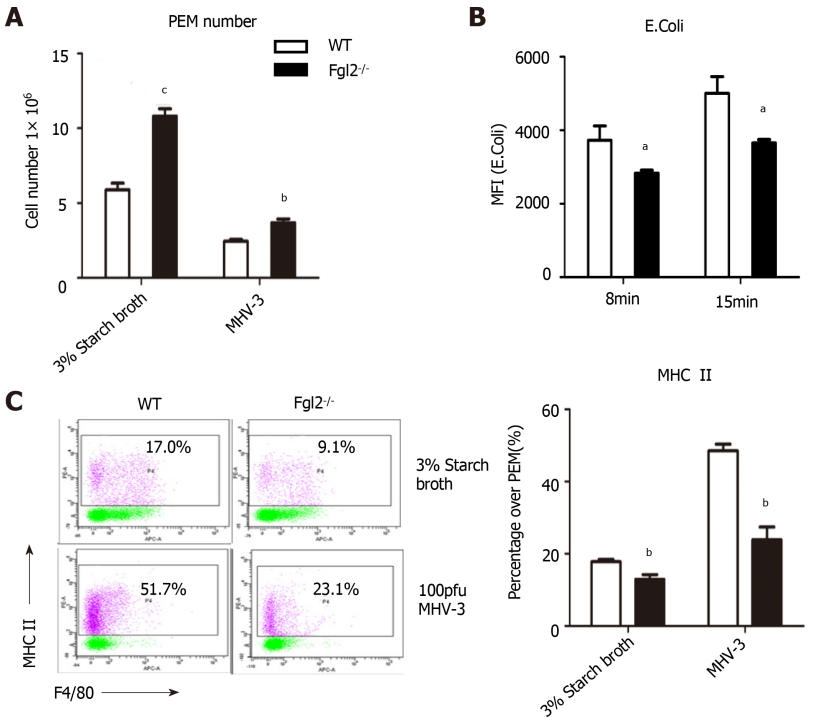


Figure 7 Fibrinogen-like protein 2 deficiency resulted in low expression of major histocompatibility complex II and impaired phagocytosis of macrophage. A: Absolute number of PEMs under the stimulation of 3% starch broth or murine hepatitis virus strain 3 (MHV-3) extracted from wild-type (WT) and fibrinogen-like protein 2 (*Fgl2-/-*) mice; B: MFI of FITC+ phagocyted *E.Coli* by PEMs from WT and *Fgl2-/-* mice at different time points; C: Expression of major histocompatibility complex II on WT and *Fgl2-/-* PEMs under the stimulation of 3% starch broth or MHV-3 infection. All data are presented as mean ± SD. These experiments were repeated at least three times. PEMs: Peritoneal exudate macrophages.

affected in *Fgl2-/-* BMDMs compared with those in WT BMDMs following LPS treatment and viral infection (Figure 8C, Supplementary Figure 5), suggesting that FGL2 induces a positive feedback proinflammatory loop through p38 activation. Phosphorylation of p65 at Ser276 by c-Raf1 enables the recruitment of acetyltransferase CREB-binding protein (CBP) and p300, which leads to the acetylation of p65 and thus an enhanced NF-κB transcription rate[16]. Interestingly, phosphorylation of c-Raf1 and p65 (Ser276) and acetylation of p65 were reduced in *Fgl2-/-* BMDMs compared with those in WT BMDMs following either LPS stimulation or viral infection (Figure 8D). Phosphorylation of TBK, a modulator downstream of SYK that is involved in C-type lectin receptor (CLR)/Toll-like receptor (TLR) signaling-mediated innate immunity [25], did not differ between WT and *Fgl2-/-* BMDMs following LPS and MHV-3 challenge (Figure 8E). Taken together, our data suggest that induction of FGL2 in macrophages regulates inflammatory signaling by modulating p38, IRF3, IRF7, and p65 phosphorylation.

DISCUSSION

Fulminant hepatitis, the most severe form of acute viral hepatitis, is a type of ALF induced by viral infection. Mounting evidence suggests that leukocyte infiltration initiates inflammation and liver damage in chronic viral hepatitis and alcoholic and non-alcoholic hepatitis with acute liver injury. However, there is no ideal experimental model to completely mimic the specific clinical manifestations of acute viral hepatitis. Most studies to date have focused on acute hepatitis B, in which virus-specific cytotoxic T lymphocytes (CTLs) mediate liver damage subsequent to viremia[26,27]. Similarly, non-virus-specific CD8+ T cells with innate-like cytolytic activity promote liver damage in patients with acute hepatitis A[28]. The innate immune response is involved in the early stage of viral clearance. Among innate immune cells, natural killer (NK) cells are preferentially studied because of their potent capacity to direct viral clearance as well as liver injury. Evidence from experimental VFH models and patients with acute viral hepatitis has revealed the detrimental contribution of NK cells to disease outcome[29,30]. Macrophages are the largest population of non-parenchymal immune cells in the liver, which proliferate and are activated in patients

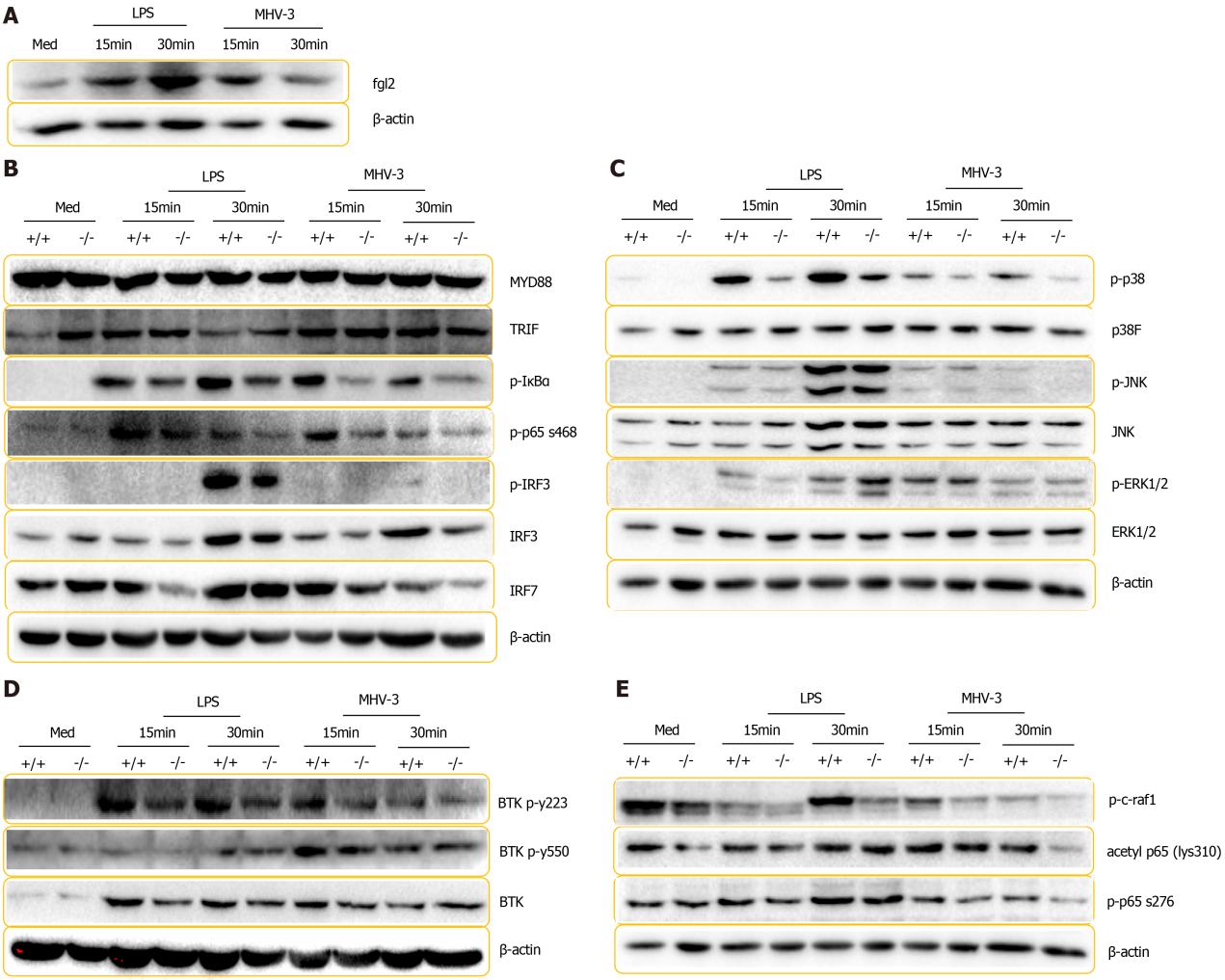


Figure 8 Fibrinogen-like protein 2 deficiency impaired inflammatory cascade in response to lipopolysaccharide treatment of viral infection. A: Immunoblot for fibrinogen-like protein 2 (Fgl2) and β-actin expression in wild-type (WT) BMDMs in response lipopolysaccharide (LPS) stimulation and murine hepatitis virus strain 3 (MHV-3) infection. B-E: Immunoblot for proteins panels from lysates of WT and *Fgl2-/-* BMDMs in response to LPS treatment and MHV-3 infection; B: MyD88, TRIF, IκBα, phosphorylated IκBα, phosphorylated p65 (S468) and IRF3, phosphorylated IRF3, and IRF7; C: p38, JNK, ERK and phosphorylated p38, JNK, ERK; D: Phosphorylated c-Raf, phosphorylated p65 (S276), acetyl p65 (lys310); E: BTK, and phosphorylated BTK (Y223), BTK (Tyr550). These experiments were repeated at least three times.

with acute hepatitis E virus infection and associated ALF, although their function is somewhat impaired[31]. The development of VFH is associated with hepatic macrophage replenishment by infiltrating macrophages, suggesting that macrophage infiltration is a fundamental event in VFH progression[32]. However, the pathological involvement of innate immunity in the liver damage occurring during acute viral hepatitis is not fully understood.

Experimental VFH established by either the coronavirus MHV-3 or infection with other hepatitis-causing viruses induces acute liver injury followed by ALF in 3-5 d in different strains of mice[12,14]. This suggests that adaptive immunity may not be the central event in disease progression, because of the rapid mortality despite infiltration of T cells to the liver[33]. In addition to monocytes, dendritic cells and neutrophils are also recruited to the liver during the progression of VFH, although the characteristics of the infiltrating leukocytes and their respective contributions to inflammation are unclear[12]. Neutrophils are a small subset of leukocytes that are required for monocyte infiltration during liver injury in mouse models of non-alcoholic steatohepatitis, acetaminophen overdose, and hepatitis virus infection[9,12,24]. In this study, we found that MoMFs dominated the population of infiltrating leukocytes, with a proinflammatory Ly6C^{high} MoMF subset comprising the majority of the population, suggesting that targeting the transition from Ly6C^{high} MoMFs to Ly6C^{low} MoMFs may be a promising strategy for treating VFH. Notably, the continued decrease in KC numbers during VFH suggests that necrosis occurred not only in hepatocytes but also in non-parenchymal cells.

FGL2 deficiency has been shown to prevent fulminant hepatitis following MHV-3 infection, which otherwise causes ALF with 100% mortality[14]. Moreover, FGL2 expression is largely distributed in and robustly induced by macrophages, in spite of moderate expression in dendritic cells and endothelial cells following viral infection [7]. The mechanism by which FGL2 contributes to VFH is generally considered to involve its procoagulant activity[14,34]. However, reciprocal activation between inflammation mediators or cytokines and tissue factor-mediated fibrin deposition and thrombin generation, the major contributor to inflammation-initiated coagulation, is largely dependent on IL-6[11,35]. It is therefore difficult to determine whether co-localization between fibrin deposition and FGL2 expression solely results from the prothrombinase activity of FGL2. In addition, inflammation may be induced by tissue factors secreted by local endothelial and immune cells[36]. Proinflammatory macrophage polarization is a process resulting from the Th1 response and pattern recognition receptor (PRR)-mediated signaling events initiated by PAMPs or DAMPs [37]. Unlike soluble FGL2 expressed by regulatory T cells and Th2-like immune cells with immunosuppressive activity, membrane FGL2 can amplify the classical inflammatory cascades and may act as a co-receptor to cooperate with TLRs or other PPRs for signal transduction. Our results suggest that FGL2 deficiency may foster a default transition from proinflammatory macrophages to an alternative activated phenotype because more M2 macrophages and Ly6C^{lo} MoMFs were observed under normal conditions. The inflammatory cytokines secreted by endothelial and dendritic cells in which FGL2 is expressed may also play a role; however, compared to macrophages, these cells are in relatively low abundance[38].

The innate immune responses triggered by viral infection are largely regulated by PPR-mediated signaling, which depends on cytosolic adaptors (*e.g.*, TRIF and MyD88) and kinase-dependent factors[39]. Macrophage M1 polarization promotes the activation and translocation of NF-κB and the IRF3/IRF7 complex to initiate the transcription of Th1-associated gene targets[40]. p38 MAPKs are known to modulate PPR signaling by collaborating with NF-κB-mediated transcription[33]. CLRs are another type of PPR that modulate TLR-mediated signaling in the defense against invading pathogens[14]. In invertebrates, numerous molecules containing fibrinogen-related domains participate in immune response transcription and have been shown to act as PPRs in defense processes, such as agglutination, and to cooperate with CLRs to synergistically clear pathogens[41]. Thus, we questioned whether FGL2 regulated TLR signaling in a CTL-like manner. MyD88-dependent PPR signaling involves activation of members of the IRAK family, which in turn stimulates the E3 ligase activity of TRAF6, enabling activation of the downstream ubiquitin-dependent kinase TAK1[42]. Upon activation, TAK1 activates MAPK and its downstream kinase IKK, which in turn phosphorylates the NF-κB inhibitor IκBα, leading to ubiquitin-dependent IκBα degradation and subsequent NF-κB activation[42]. Thus, TRIF-dependent PPR signaling involves TRAF3 recruitment and activation of TBK1 and IKK□, which stimulate IRF3 phosphorylation and NF-κB activation, thereby leading to the transcriptional induction of type I IFNs and inflammatory cytokines[43]. Membrane proteins that regulate membrane PPR-mediated signaling function as co-receptors or factors involved in TLR assembly, internalization, or trafficking[44-47]. Accordingly, FGL2 may act as an assembly factor, could interact with PPRs for ligand-receptor binding, or even mediate the trafficking between the membrane and endosome, thereby facilitating inflammatory signaling transduction. Previous data showed that MHV-3-induced FGL2 expression in macrophages relies on p38 activation[48]. In this work, we found that FGL2 is needed for p38 phosphorylation, suggesting that FGL2 expression provides a positive feedback loop for proinflammatory aggregation in macrophages.

The exact role of resident macrophages and MoMFs in VFH is obscure. We speculate that resident macrophages may initiate liver inflammation during the early stage of viral infection because MoMFs exacerbate liver damage during the acute phase. Resident macrophages represent the first line of defense against viral infection. Once primed, resident macrophages produce chemotaxins and cytokines that recruit and activate the MoMFs, and our results suggest that FGL2 expression on macrophages prompts and enhances their inflammatory phenotype.

CONCLUSION

Our data revealed, for the first time, that Ly6C^{high} MoMF infiltration is a critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-

induced hepatitis progression. FGL2 expression is required for maintaining the proinflammatory Ly6C^{high} MoMF phenotype by mediating IRF3, p65, and p38 phosphorylation, thereby forming a positive feedback loop of inflammatory accumulation in the liver.

ARTICLE HIGHLIGHTS

Research background

Viral fulminant hepatitis (VFH) is a devastating syndrome that pathologically caused by excessive activation of both innate and adaptive immunity. However, the extent of contribution of innate immunity in VFH is not well defined. Macrophage polarization have been implicated in host defense and the pathogenesis of various hepatic diseases. Fibrinogen-like protein 2 (FGL2) can be induced robustly and exclusively in macrophages in response to cytokines or viral infection. Exploring their roles in VFH can greatly improve our understanding of the disease and thus seek the therapeutic approaches.

Research motivation

Hepatic macrophages are attractive therapeutic targets because their functions can be inhibited or augmented to alter disease outcomes. A better understanding of their biological properties and immunologic function in liver homeostasis and pathology may pave the way for new diagnostic and therapeutic approaches for liver failure or other liver diseases.

Research objectives

To evaluate the role of Fgl2 in the reconstitution of hepatic macrophages during VFH progression by the VFH mouse model.

Research methods

Liver sections of liver failure patients and controls were immuno-stained for macrophages examination. Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH experimental model in wild type and *Fgl2*-/- mice. Adoptive transfer or depletion of macrophages were employed to assess liver damage and hepatic macrophages alteration. Signal cascade induced by LPS or MHV-3 were detected in macrophages.

Research results

Infiltrated MoMFs is a major source of hepatic inflammation during VFH progression. Fgl2 expression on macrophages prompts and enhances the inflammatory phenotype of hepatic macrophages, which breaks the previous understanding that the mechanism of Fgl2 during VFH is generally considered to be procoagulant activity.

Research conclusions

We revealed for the first time that pro-inflammatory monocyte-derived macrophages (MoMFs) infiltration is critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-induced hepatitis progression and Fgl2 is required for maintaining the pro-inflammatory hepatic macrophages phenotype.

Research perspectives

Macrophages are 'keystones' of liver architecture in both homeostasis and disease. The development of potential therapies highly depends on a fundamental knowledge about the mechanisms that trigger the polarization and control the fate of hepatic macrophages. We believe that a better understanding of the specific mechanisms underlying macrophages participation in diseases will definitely result in the increased efficacy of these therapies.

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LETTER TO THE EDITOR

Interplay between chronic hepatitis B and atherosclerosis: Innovative perspectives and theories

Ivan Ranković, Vladimir Milivojević, Aleksandra Pavlović Marković, Mihailo Bezmarević

ORCID number: Ivan Ranković 0000-0002-5767-3914; Vladimir Milivojević 0000-0003-3601-7528; Aleksandra Pavlović Marković 0000-0002-7899-5322; Mihailo Bezmarević 0000-0003-1938-8298.

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Ivan Ranković, Clinic for Gastroenterology and Hepatology, University Clinical Center of Serbia, Belgrade 11000, Serbia

Vladimir Milivojević, Aleksandra Pavlović Marković, Clinic for Gastroenterology and Hepatology, University Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade 11000, Serbia

Mihailo Bezmarević, Department of Hepatobiliary and Pancreatic Surgery, Clinic for General Surgery, Military Medical Academy, University of Defense, Belgrade 11000, Serbia

Corresponding author: Mihailo Bezmarević, MD, MSc, Doctor, Senior Researcher, Surgical Oncologist, Department of Hepatobiliary and Pancreatic Surgery, Clinic for General Surgery, Military Medical Academy, University of Defense, Crnotravska 17, Belgrade 11000, Serbia. bezmarevicm@gmail.com

Abstract

Elaboration of carotid atherosclerosis in the setting of hepatitis B virus (HBV) infection should emphasize the significance of extrahepatic manifestations of the infection pathogenesis. Diverse processes comprise the pathoevolution of HBV infection, rendering it a multi-systemic disease in its essence. Our work not only exemplified atherosclerosis as an often-underestimated contributor to the severity of HBV infection but has also highlighted the bidirectional relationship between the two. Therefore, it is suggested that HBV-induced inflammation is one of the root causes of atherosclerosis, which in turn has a consequent effect on the severity of the chronic infection disease state, creating a vicious cycle. Additionally, we coupled prior data with the current concepts of HBV infection to postulate intriguing perspectives and theories.

Key Words: Hepatitis B virus infection; Carotid atherosclerosis; Hepatitis B e antigen; Arterial inflammation; Perspectives and trends

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Core Tip: Hepatitis B virus (HBV) infection is a multifaceted disease, with significant cardiovascular morbidity. Our innovative approach to this pathophysiologic

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relationship harbors several key ideas. First, HBV infection may carry a specific atherosclerosis distribution pattern, with predilection for carotid arteries. Second, we propose wider use of more sensitive inflammatory markers, such as high-sensitivity C-reactive protein and homocysteine. Third, macrophage phenotype function should be investigated, utilizing its potential role as an atherosclerosis biomarker in HBV infection and therapeutic target. Last but not least, we reason that statins should be exploited more in current practice, due to their favorable pleotropic effects.

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TO THE EDITOR

We read with interest the study of Riveiro-Barciela *et al*[1] which elucidates the possible interplay between hepatitis B virus (HBV) infection and carotid atherosclerosis. It has high-yield trial properties due to its large sample size and prospective method. Although its inclusion and exclusion criteria were precise and broad, as with any case-control study, there remains the possibility of bias as a consequence of inferring causation from statistically significant correlations which can be complicated by difficulty in determining the chronological order of exposure to HBV (*i.e.* the starting time of infection and latency).

The authors concluded that the presence of subclinical atherosclerosis and carotid plaques were more frequent in patients with HBV infection than in controls and that liver damage was an independent factor associated with subclinical atherosclerosis and carotid plaques, regardless of the presence of classical cardiovascular factors.

In general, we agree with the authors, since many of our patients render a similar atherosclerotic disease profile which cannot be attributed solely, *sui generis*, to the cardiovascular substrate. Therefore, their study's findings have the capacity not only to raise the index of suspicion of a practicing clinician but to optimize the established diagnostic framework of HBV patients in order to prevent atherosclerosis occurrence and complications.

Furthermore, the study implicates chronic HBV infection (*i.e.* the specific point of the naïve hepatitis B e antigen (HBeAg)-negative phase) as being an important atherosclerotic contributor. Conversely, a prior study by Tong *et al*[2] has concluded that HBV infection not only negatively correlates with C-reactive protein (CRP) levels but seems to not be associated with coronary atherosclerosis. Additionally, Kiechl *et al*[3] found no significant association between chronic hepatitis and the development of new carotid atherosclerotic plaques; although, they did not specify the type of hepatitis virus. Of course, these conflicting results have to be considered cautiously, as they originate from patients in different phases of the HBV infection and divergent research materials and methods. With all this said, it may be that the window of opportunity for early atherosclerosis detection and preemptive therapeutic intervention in HBV could represent the subpopulation of naïve and HBeAg-negative patients.

However, the general discrepancies in the conclusions of the aforementioned trials made us postulate some intriguing perspective theories. First, it may be that HBV infection harbors specific propensity towards anatomically different vascular structures, thereby affecting carotid arteries more often than coronary arteries. This notion is in concordance with the previously published data inferring that viruses have different sites of endothelial predilection[4]. Second, we may utilize other, more sophisticated inflammatory markers, namely high-sensitivity (hs-)CRP with or without homocysteine for optimal HBV patient stratification regarding atherosclerosis risk[5]. Third, the potential role of macrophage phenotype variation during HBV infection may be one of the crossroads between the processes of atherosclerosis and HBV infection[6]. Current cardiology investigations have revealed the significant role of macrophages, encompassing their local, endothelial, as well as systemic effects *via* T-helper lymphocytes and cytokine release modulation[7]. Having stated that, we

postulate that HBV infection may trigger macrophage phenotype alteration, rendering it to be a contributive precipitant of atherosclerotic disease as well as the crosslink point between the two diseases. Last but not least, the study of Riveiro-Barciela *et al*[1] may open the door for broader statin use, addressing two end goals concomitantly: Lowering the risk of cirrhosis and hepatocellular carcinoma in viral hepatitis patients [8], and engaging in the prevention and treatment of atherosclerosis and its complications.

We believe that prospects in this field should be diversified in the manner that one-size does not fit all. Upcoming trials and future viewpoints should render better comprehension of the delicate HBV pathodynamics from which implementation of optimized and specific therapy would be more feasible.

Our group envisions many possible pathways between HBV infection and atherosclerosis, *i.e*. cardiovascular diseases which may be potential targets for clinical management, and thus encourages future research work in this field.

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LETTER TO THE EDITOR

Is CA19-9 effective in predicting chemotherapeutic response in patients with synchronous liver metastases with colorectal cancer?

Semra Demirli Atici, Erdinc Kamer

ORCID number: Semra Demirli Atici 0000-0002-8287-067X; Erdinc Kamer 0000-0002-5084-5867.

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Semra Demirli Atici, Erdinc Kamer, Department of General Surgery, University of Health Sciences Tepecik Training and Research Hospital, İzmir 35180, Turkey

Corresponding author: Semra Demirli Atici, MD, Surgeon, Department of General Surgery, University of Health Sciences Tepecik Training and Research Hospital, Güney Mahallesi, 1140/1 Sokak No:1 Yenişehir/Konak, İzmir 35180, Turkey. smrdemirli@hotmail.com

Abstract

Evaluation of response to chemotherapy in colorectal cancer patients with synchronous liver metastases is important in terms of treatment management. In this Letter to the Editor, several issues in the article are discussed. For the comparison of carbohydrate antigen 19-9 (CA19-9) values referenced in the study, the patient group was not matched for cancer stage. Therefore, it may be more appropriate to select and compare CA19-9 values in patients with same-stage cancer.

Key Words: Colorectal cancer; Carbohydrate antigen 19-9; Liver metastasis of colorectal cancer; Synchronous liver metastasis; Chemotherapy; Metastatic colorectal cancer

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Core Tip: It is important to evaluate synchronous liver metastases of colorectal cancer (CRC) and to determine the response to chemotherapy in patients. Based on findings from such, the optimal treatment method is selected for each patient. The scoring system described in the study, created through a combination of radiology and laboratory parameters, can guide treatment. However, we think that it would be more appropriate to discuss the results of this study in the context of other studies conducted with patients with stage IV CRC.

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TO THE EDITOR

Ma *et al*[1] recently published a retrospective study on the emerging role of a magnetic resonance imaging (MRI)-radiomics signature to detect the predictive efficiency of models for chemotherapeutic response in colorectal cancer (CRC) patients with synchronous liver metastasis (SLM) and avoid ineffective chemotherapy.

Carbohydrate antigen 19-9 (CA19-9) has been routinely studied in patients with CRC, and in the study by Ma *et al*[1] the measurement of CA19-9 was found to be significant between the disease non-response (non-DR) and disease response (DR) to chemotherapy groups (P = 0.045). The authors showed that CA19-9 Levels were higher in the DR (63.3%) group than in the non-DR group (43.4%). The authors reported that CA19-9 is a promising indicator for predicting response to chemotherapy, citing the study by Zhou *et al*[2]. However, the study design used by Zhou *et al*[2] had included patients with stage III CRC, while the study by Ma *et al*[1] focused on patients with stage IV CRC.

Although it is known that high CA19-9 Levels are a poor-prognosis factor in untreated stage IV CRC patients, routine measurement of CA19-9 in colon cancers is not recommended by the American Society of Clinical Oncology (ASCO) guidelines due to insufficient evidence[3,4]. As such, we believe that it would be more appropriate to discuss the results of the study by Ma *et al*[1] in the context of other studies conducted with stage IV CRC patients[4,5].

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Contents

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FRONTIER

502 Colorectal cancer screening and surveillance in patients with inflammatory bowel disease in 2021

Huguet JM, Ferrer-Barceló L, Suárez P, Sanchez E, Prieto JD, Garcia V, Sempere J

MINIREVIEWS

517 Viral hepatitis: Innovations and expectations

Leoni S, Casabianca A, Biagioni B, Serio I

ORIGINAL ARTICLE

Basic Study

532 Effect of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* supernatants on serotonin transporter expression in cells and tissues

Chen YM, Li Y, Wang X, Wang ZL, Hou JJ, Su S, Zhong WL, Xu X, Zhang J, Wang BM, Wang YM

547 Connective tissue growth factor expression hints at aggressive nature of colorectal cancer

Bhat IP, Rather TB, Maqbool I, Rashid G, Akhtar K, Bhat GA, Parray FQ, Syed B, Khan IY, Kazi M, Hussain MD, Syed M

Retrospective Cohort Study

570 Abnormal liver chemistries as a predictor of COVID-19 severity and clinical outcomes in hospitalized patients

Krishnan A, Prichett L, Tao X, Alqahtani SA, Hamilton JP, Mezey E, Strauss AT, Kim A, Potter JJ, Chen PH, Woreta TA

CASE REPORT

588 Simultaneous endoscopic and video-assisted retroperitoneal debridement in walled-off pancreatic necrosis using a laparoscopic access platform: Two case reports

Lindgaard L, Lauritsen ML, Novovic S, Hansen EF, Karstensen JG, Schmidt PN

594 Curative resection with endoscopic submucosal dissection of early gastric cancer in *Helicobacter pylori*-negative Ménétrier's disease: A case report

Fukushi K, Goda K, Kino H, Kondo M, Kanazawa M, Kashima K, Kanamori A, Abe K, Suzuki T, Tominaga K, Yamagishi H, Irisawa A

LETTER TO THE EDITOR

602 Artificial intelligence model validation before its application in clinical diagnosis assistance

Vazquez-Zapien GJ, Mata-Miranda MM, Garibay-Gonzalez F, Sanchez-Brito M

605 Machine learning models and over-fitting considerations

Charilaou P, Battat R

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Contents

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Editorial Board Member of *World Journal of Gastroenterology*, Guang Ji, MD, PhD, Professor, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, No. 725 South Wanping Road, Shanghai 200032, China. jiliver@vip.sina.com

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FRONTIER

Colorectal cancer screening and surveillance in patients with inflammatory bowel disease in 2021

Jose Maria Huguet, Luis Ferrer-Barceló, Patrícia Suárez, Eva Sanchez, Jose David Prieto, Victor Garcia, Javier Sempere

ORCID number: Jose Maria Huguet 0000-0001-6486-1262; Luis Ferrer-Barceló 0000-0001-8372-1572; Patrícia Suárez 0000-0001-9306-8378; Eva Sanchez 0000-0003-2489-5366; Jose David Prieto 0000-0002-7519-3893; Victor Garcia 0000-0002-4662-2070; Javier Sempere 0000-0002-6893-2512.

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Jose Maria Huguet, Luis Ferrer-Barceló, Patrícia Suárez, Eva Sanchez, Jose David Prieto, Victor Garcia, Javier Sempere, Department of Digestive Disease, General University Hospital of Valencia, Valencia 46014, Spain

Corresponding author: Jose Maria Huguet, MD, PhD, Assistant Professor, Department of Digestive Disease, General University Hospital of Valencia, Av Tres Cruces 2, Valencia 46014, Spain. josemahuguet@gmail.com

Abstract

The detection of dysplasia in patients with inflammatory bowel disease (IBD) continues to be important given the increased risk of colorectal cancer in this population. Therefore, in 2017, we performed a review and update of the recommendations for the management and follow-up of patients with IBD based on the clinical practice guidelines of various scientific societies. The present manuscript focuses on new aspects of the detection, follow-up, and management of dysplasia according to the latest studies and recommendations. While chromoendoscopy with targeted biopsy continues to be the technique of choice for the screening and detection of dysplasia in IBD, the associated difficulties mean that it is now being compared with other techniques (virtual chromoendoscopy), which yield similar results with less technical difficulties. Furthermore, the emergence of new endoscopy techniques that are still being researched but seem promising (*e.g.*, confocal laser endomicroscopy and full-spectrum endoscopy), together with the development of devices that improve endoscopic visualization (*e.g.*, Endocuff Vision), lead us to believe that these approaches can revolutionize the screening and follow-up of dysplasia in patients with IBD. Nevertheless, further studies are warranted to define the optimal follow-up strategy in this patient population.

Key Words: Colitis surveillance; Colitis screening; Chromoendoscopy; Colorectal cancer; Inflammatory bowel disease; Ulcerative colitis

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Core Tip: Patients with inflammatory bowel disease (IBD) are at greater risk of

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colorectal cancer over time. Therefore, a series of recommendations has been made for the follow-up of this population. We carried out a review in which we set out the main new developments in the detection, follow-up, and management of dysplasia in patients with IBD in recent years.

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INTRODUCTION

The worldwide consensus among scientific societies is that screening for colorectal cancer (CRC) in patients with inflammatory bowel disease (IBD)[1-5] should be carried out by means of colonoscopy, preferably during the remission phase and with appropriate bowel preparation[6-10]. The objective is to detect potentially resectable premalignant lesions (dysplasia) and CRC in the early stages, thus improving prognosis[11-15]. While CRC-related mortality has decreased since the introduction of endoscopic screening techniques[16-19], the risk of CRC remains unchanged[20].

The differences in the recommendations of scientific societies on screening and surveillance in affected patients led us to review and update the recommendations of the various scientific societies and research groups in 2017[21]. Nevertheless, novel aspects and updates that have appeared since then are worthy of review, even though research in some areas remains incomplete.

SUMMARY OF OUR RECOMMENDATIONS

Below is a summary of our previously published recommendations

To whom should CRC screening be offered? Screening for CRC should be offered to the following patients affected by IBD: patients with ulcerative colitis (UC), regardless of its extent; patients with Crohn's disease (CD) affecting at least one-third of the colon or accompanied by complex perianal disease; patients with an ileoanal pouch; and patients with indeterminate or unclassified colitis. Endoscopy should be performed preferably in periods of clinical-biological remission and should enable an estimation of the individual risk of CRC, as well as the extent of the disease[21].

When should the first colonoscopy screening be performed? The first colonoscopy screening should be offered eight years after a diagnosis of CD or UC. In patients with primary sclerosing cholangitis (PSC), colonoscopy should be performed as soon as possible. In patients with an ileoanal pouch, colonoscopy should be performed one year after the surgical intervention. Patients with first-degree relatives who were diagnosed with CRC before age 50 should be offered the first endoscopy ten years before the age the family member was when affected by CRC or eight years after a diagnosis of IBD (whichever occurs earlier).

Who should be offered endoscopic surveillance? After screening, all patients should undergo endoscopy-based follow-up, except for those with ulcerative proctitis, CD with the involvement of less than one-third of the colon, and those in whom the risks outweigh the possible benefits.

Should the same endoscopic surveillance intervals be followed for all patients? No. We recommend that patients be stratified according to their individual risk.

Are there individual risk factors that allow us to stratify endoscopic surveillance? Yes. Among patients with left-sided UC or pancolitis and CD affecting at least one-third of the colon, high-risk patients are defined as those having any of the following: PSC, extensive involvement, moderate-severe active inflammation sustained over time (endoscopic or histological), a first-degree relative with CRC before age 50, or stenosis

or dysplasia detected during the previous five years. Intermediate-risk patients are defined as those having any of the following: extensive colitis with mild or moderate sustained active inflammation (endoscopic or histological), inflammatory polyps, or a first-degree relative with CRC after age 50. A diagnosis of IBD at a young age should be taken into account as a relative risk factor (due to the long duration of the disease). Factors other than high- and intermediate-risk factors should be considered low-risk factors. High-risk factors in patients with an ileoanal pouch are as follows: dysplasia or previous CRC, PSC, and type C mucosa in the pouch (persistent atrophy and severe inflammation) (Table 1).

How long should the endoscopic follow-up intervals be? Endoscopic follow-up intervals are recommended as follows: for patients with IBD, according to the presence of risk factors for each patient; for patients with high-risk factors, annual colonoscopy; for patients with intermediate-risk factors, colonoscopy every three years; for patients with low-risk factors or no other risk factors, colonoscopy every five years; for patients with an ileoanal pouch, according to the presence of risk factors; for patients with risk factors, annual colonoscopy; and for patients with no risk factors, colonoscopy every five years (Table 1).

What is the recommended endoscopic technique for screening and surveillance? Chromoendoscopy with endoscopic resection or biopsies aimed at visible lesions is the technique of choice. If this is not possible, high-definition video colonoscopy should be used, and four biopsies should be taken every ten cm of the colon.

How is chromoendoscopy performed? Methylene blue (0.04%-0.1%) or indigo carmine (0.1%-0.03%) is used. Caecal intubation is performed, and a dye is applied to the mucosa of the colon as the endoscope is removed, if possible, using a catheter spray. One segment should be examined before applying a dye to the next segment.

Does the occurrence of dysplasia require confirmation? The occurrence of dysplasia must be confirmed by a second pathologist.

What terminology should be used to describe lesions detected with endoscopy? The terms "dysplasia-associated lesion or mass (DALM)" and "flat lesions" should be discontinued. The modified Paris Classification, in which lesions are divided into visible dysplasia and invisible dysplasia depending on whether the biopsy has been taken from a lesion visualized in the colonoscopy or not, should be used. Visible dysplasia is divided into polypoid and non-polypoid depending on whether the lesion protrudes from the lumen ≥ 2.5 mm. The descriptions of visible lesions should also include a mention of whether they are ulcerated and whether the borders are easily distinguished from the surrounding mucosa (Table 2).

How should a visible lesion be managed? Visible lesions that are well delimited, with no evidence of dysplasia in the mucosa adjacent to the lesion and no synchronous dysplasia, should be resected endoscopically regardless of the degree of dysplasia.

How should invisible dysplasia be initially managed? Dysplasia that is not endoscopically visible but found in serial biopsies of the colon must be confirmed by an independent pathologist after chromoendoscopy is performed by an expert endoscopist. If dysplasia is confirmed, management will depend on the degree.

How should invisible dysplasia be managed in relation to the degree of dysplasia? Endoscopically invisible high-grade dysplasia is an indication for colectomy. The management of low-grade, invisible dysplasia should be agreed upon in a multidisciplinary committee and with the patient: the two possible options are colectomy and endoscopic follow-up.

How should endoscopically resected lesions be followed? The follow-up for resected lesions in healthy mucosa not affected by colitis should be the same as that for sporadic adenomas. Lesions that are endoscopically resected in areas affected by colitis should be examined endoscopically at three months and annually thereafter.

NOVEL ASPECTS IN SCREENING AND FOLLOW-UP

The most recent updates to clinical practice guidelines[22-24] do not contain substantial modifications with respect to candidates for screening, the periodicity of

Table 1 Risk factors for the development of colorectal cancer in patients with inflammatory bowel disease and recommended surveillance

	High risk	Intermediate risk	Low risk
Risk factors	(1) PSC; (2) Extensive involvement; (3) Moderate-severe active inflammation sustained over time (endoscopic or histological); (4) First-degree relative with CRC before age 50; (5) Stenosis or dysplasia detected during the previous five years; (6) Appearance of IBD at a young age; (7) If ileo-anal pouch: (a) Dysplasia; (b) Previous CRC; (c) PSC; and (d) Type C mucosa in the pouch	(1) Extensive colitis with mild or moderate sustained inflammatory activity (endoscopic or histological); (2) Inflammatory polyps; and (3) First-degree relative with CRC after age 50	(1) Factors other than high and intermediate risk; and (2) If ileo-anal pouch: Without risk factors
Surveillance	Annual	Every three years	Every five years

CRC: Colorectal cancer; IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis.

Table 2 SCENIC international consensus

Term	Definition
Visible dysplasia	Dysplasia identified on targeted biopsies from a lesion visualized in colonoscopy
Polypoid	Lesion protruding from the mucosa into the lumen ≥ 2.5 mm
Pedunculated	Lesion attached to the mucosa by a stalk
Sessile	Lesion not attached to the mucosa by a stalk: entire base is contiguous with the mucosa
Nonpolypoid	Lesion with little (< 2.5 mm) or no protrusion above the mucosa
Superficially elevated	Lesion with protrusion but < 2.5 mm above the lumen (less than the height of the closed cup of a biopsy forceps)
Flat	Lesion without protrusion above the mucosa
Depressed	Lesion with at least a portion depressed below the level of the mucosa
General descriptors	
Ulcerated	Ulceration (fibrinous base with depth) within the lesion
Border	
Distinct border	Border of the lesion is discrete and can be distinguished from surrounding mucosa
Indistinct border	Border of the lesion is not discrete and cannot be distinguished from surrounding mucosa
Invisible dysplasia	Dysplasia identified on random (non-targeted) biopsies of colon mucosa without a visible lesion

screening, or risk factors for CRC in these patients. Similarly, no novel aspects in this regard have been published in recent reviews[25]. While neither new research nor changes in current knowledge are expected in this field, it is worth taking into account the observation made by Burke *et al*[26], who concluded that patients with IBD and high-risk factors should be closely followed up at short intervals. These authors performed a retrospective review of interval CRC associated with IBD and not associated with IBD diagnosed between January 2007 and December 2014 in a large-scale American health system. When they compared cases of interval CRC associated and not associated with IBD, they found that associated cases were more common in younger patients (54.5 *vs* 70.4 years; $P < 0.001$); these had appeared within a shorter interval after the index colonoscopy (20.7 *vs* 35.1 mo; $P = 0.09$). An evaluation of adherence to American Society for Gastrointestinal Endoscopy guidelines revealed that 53% (8/15) of cases of interval CRC in patients with IBD were detected in line with surveillance guidelines. All of the patients with interval CRC detected after the surveillance period recommended in the guidelines had high-risk factors during the index colonoscopy, namely, active inflammation, multiple pseudopolyps, previous low-grade or indefinite dysplasia, or a family history (first-degree) of CRC[26].

A recent study analysed 9398 pouchoscopy procedures performed in 3672 patients and concluded that the low incidence rate for neoplasm recorded suggests that pouchoscopy was not routinely necessary in asymptomatic patients, in patients with a history of primary sclerosing cholangitis, or in patients with chronic pouchitis, although it is recommended when there is a personal or family history of CRC. More evidence is necessary before guidelines can be changed[27].

NOVEL ASPECTS OF THE RECOMMENDED ENDOSCOPIC TECHNIQUE

Up to the time of our review[21], there was a general consensus on using chromoendoscopy with methylene blue or indigo carmine as the technique of choice, mainly since the publication of the SCENIC CONSENSUS[28]. In situations where chromoendoscopy was not possible, the technique of choice was high-definition video colonoscopy and serial biopsies every ten cm of the colon. New subsequent guidelines maintained this recommendation[29], and real-life studies supported the decision[30].

However, the recommendation soon began to be called into question, and the new ACG guidelines stated the following: *When using high-definition colonoscopes in patients with UC undergoing surveillance, we suggest white-light endoscopy with narrow-band imaging or dye-spray chromoendoscopy with methylene blue or indigo carmine to identify dysplasia (conditional recommendation, low quality of evidence)*[23]. Furthermore, the difficulties arising with chromoendoscopy were reviewed by our group and included a long examination time, the need for optimal bowel preparation, specialist training, and higher costs[31]. These difficulties could have led to chromoendoscopy being performed less frequently than desired, despite recommendations from scientific societies on the application of the technique. Such were the findings of a recent online survey administered to academic gastroenterologists belonging to the Canadian Association of Gastroenterology, which revealed low uptake of chromoendoscopy as a surveillance tool for dysplasia in patients with IBD. The main barriers reported were the long duration of the procedure, cost, and the lack of experience or training in the technique[32].

The situation was recently evaluated by Bisschops *et al*[33], who performed a multicentre prospective randomized controlled trial of 131 patients with UC and found that chromoendoscopy and virtual chromoendoscopy (VCE) with narrow-band imaging (NBI) presented no significant differences for the detection of dysplasia or neoplasms associated with UC. Furthermore, the authors concluded that, given the reduced withdrawal time and greater ease of use, VCE with NBI should replace traditional chromoendoscopy. Clarke *et al* performed a retrospective analysis of cases and controls in which they concluded that there were no statistically significant differences in the detection of dysplasia using dye-spray chromoendoscopy compared with high-definition white-light colonoscopy, although the chromoendoscopy procedure took longer[34]. Gulati *et al*[35] performed a multifaceted randomized crossover trial to evaluate study feasibility and obtain preliminary comparative procedural and patient experience data. The authors found that the diagnostic accuracy of VCE was higher, although the differences were not statistically significant [93.7% (85.5%-98.2%) *vs* 76.9% (66.9%-85.1%) for chromoendoscopy]. In addition, the authors found that biopsy based on VCE was less frequent, with fewer dysplastic lesions overlooked than with chromoendoscopy and a failure rate of 9.1%, compared with 18.2% for chromoendoscopy. Patients generally reported a significant preference for VCE; most had a high risk of cancer requiring more intense and frequent surveillance. While the reasons for this preference were not explored, the authors suggest that it could be because the technique is less time-consuming than chromoendoscopy and there is no need for contrast or abdominal distension.

A study of 270 patients compared three different techniques for surveillance colonoscopy to detect colonic neoplastic lesions in IBD patients: high-definition, chromoendoscopy, and VCE using i-SCAN image–enhanced colonoscopy[36]. A randomized noninferiority trial was conducted to determine the detection rates of neoplastic lesions in IBD patients with longstanding colitis. The authors concluded that neither VCE nor high-definition colonoscopy was inferior to dye-spray colonoscopy for the detection of colonic neoplastic lesions during surveillance. Data from a study performed in Valencia, Spain, revealed no differences between VCE with standard i-Scan chromoendoscopy and VCE with indigo carmine 0.4% in the detection of dysplasia in the colons of patients with longstanding IBD[37]. However, VCE with i-Scan was a less time-consuming alternative. A meta-analysis from 2020 that included 17 randomized clinical trials and almost 2500 patients showed the superiority of dye-spray chromoendoscopy compared with standard-definition white-light endoscopy. No differences were detected when dye-spray chromoendoscopy was compared with high-definition white-light endoscopy or narrow-band imaging[38].

Therefore, it is very likely that we can use video chromoendoscopy as the technique of choice for the screening and follow-up of affected patients (Table 3).

Table 3 Summary of endoscopic detection techniques

Technique	Recommendation	Future
Standard-definition colonoscopy	None	No longer used
High-definition white-light video colonoscopy and serial biopsies every 10 cm of the colon	Avoid	No longer used
High-definition white-light video colonoscopy with dye-spray chromoendoscopy (methylene blue or indigo carmine)	High	Second choice
High-definition white-light video colonoscopy with narrow-band imaging	High	First choice
Full-spectrum endoscopy	Await further evidence	Under investigation
Autofluorescence imaging	None	No longer used
Confocal laser endomicroscopy	Await further evidence	Under investigation
Endocytoscopy	Investigate	Investigate

METHOD FOR TAKING BIOPSY SPECIMENS

The methods for taking targeted and serial biopsy specimens during chromoendoscopy might also be subject to change. This aspect was evaluated in a French randomized controlled trial[39], in which the authors evaluated whether taking serial biopsy specimens increased the detection rate for neoplasms in patients who had undergone screening for CRC and UC. They observed that while the yield was low, random biopsies for the detection of dysplasia should be combined with chromoendoscopy in patients with IBD and concomitant PSC, a personal history of neoplasm or the lead pipe sign. Furthermore, the rate of detection of additional neoplasms was 15% in this subgroup of patients. Along the same lines, Hu *et al*[40] recently published the results of a retrospective study of 300 patients with IBD in which they analysed the detection of dysplasia with targeted and random biopsy in 422 colonoscopies. The authors found that in up to 18% of the colonoscopies, dysplasia was identified using random biopsy. Risk factors such as concomitant PSC, a longer duration of disease, and endoscopically active disease increased the likelihood of a dysplastic lesion being detected in random biopsy, suggesting that specific high-risk patients could benefit from this strategy for the detection of dysplasia.

Previous reviews have suggested that additional biopsy specimens should be taken from the area of flat mucosa surrounding dysplastic polypoid lesions resected in IBD to confirm the absence of residual neoplasm *in situ*[28]. Nevertheless, recent studies consider these biopsies to be of low and even insignificant diagnostic yield and not predictive of findings in subsequent follow-up colonoscopy[41]. The findings reported cast doubt on the usefulness and yield of this procedure. New, high-definition techniques and chromoendoscopy may obviate the need for the biopsy of the adjacent mucosa to detect invisible dysplasia in many patients[42,43]. A recent retrospective cohort study compared the long-term effectiveness of targeted biopsy of suspected lesions with that of random biopsy and found robust evidence for targeted biopsy in the prevention of death from colon cancer. However, since panchromoendoscopy was used in only 4.6% of patients, the findings cannot be extrapolated[44].

Today, doubt remains as to which surveillance technique is best for patients with IBD and the involvement of the colon but no other risk factors for CRC: high-definition colonoscopy with random biopsy, VCE and targeted biopsy, or high-definition colonoscopy with targeted biopsy.

ENDOSCOPIC-HISTOLOGIC EVALUATION

The Paddington International Virtual ChromoendoScopy ScOre (PICaSSO) is a recently reported VCE scoring system in UC to redefine endoscopic findings of mucosal and vascular healing developed by international experts in optical diagnosis. Interobserver agreement on the pretest and the post-test evaluation was very good for the Mayo endoscopic score, Ulcerative Colitis Endoscopic Index of Severity, Robarts Histological Index, and a full spectrum of histological changes[45] (Table 4). Furthermore, a recent real-life study revealed that the PICaSSO score correlated

Table 4 The Paddington International Virtual ChromoendoScopy ScOre in ulcerative colitis

PICaSSO Mucosal Architecture	PICaSSO Vascular Architecture
0 - No mucosal defect	**0 - Vessels without dilatation**
A: Continuous/regular crypts	A: Roundish following crypt architecture
B: Crypts not visible (scar)	B: Vessels not visible (scar)
C: Discontinuous and or dilated/elongated crypts	C: Sparse (deep) vessels without dilatation
I - Micro erosion or cryptal abscess	**I - Vessels with dilatation**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	
II – Erosions, size < 5 mm	**II - Intramucosal bleeding**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	
III – Ulcerations, size > 5 mm	**III - Luminal bleeding**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	

PICaSSO: Paddington International Virtual ChromoendoScopy ScOre.

strongly with multiple histological indices and that, similar to histology, it predicted specified clinical outcomes at 6 and 12 mo. The authors concluded that PICaSSO can be a useful endoscopic tool in the therapeutic management of UC[46].

SERRATED EPITHELIAL CHANGE

In recent years, several studies have investigated the involvement of serrated epithelial changes in patients with IBD. This histopathological finding, which appears in patients with a long history of colitis in areas of nodular mucosa, is characterized by crypt distortion with diffuse striae and an epithelium rich in goblet cells. Parian *et al* recently performed a case-control study and a systematic review with a meta-analysis of 196 patients with UC in which they found higher rates of synchronous or metachronous tumours in patients with IBD and serrated epithelial change, thus leading us to believe that these patients require closer monitoring, possibly with yearly endoscopy[47].

PATIENTS WITH A LARGE NUMBER OF POLYPS IN SURVEILLANCE ENDOSCOPY

When pseudopolyps are found along an extensive area of the colon and it is impossible to remove them all appropriately to evaluate dysplasia, possible options should be discussed with the patient. Given the risk of nonidentified neoplasms, prophylactic proctocolectomy should be considered. In specific cases (patients with advanced age, high surgical risk, and refusal to undergo proctocolectomy), closer follow-up should be considered as an alternative[1,23]. With respect to this clinical situation, there have been no changes in the recommendations. This aspect should be evaluated in future studies, mainly with emerging endoscopic techniques.

INTEROBSERVER AGREEMENT IN THE HISTOLOGY ANALYSIS

The difficulties involved in interpreting histology specimens are well known with respect to the presence/absence and grade of dysplasia[48].

A recent study analysing interobserver agreement on histology findings in inflammatory bowel disease found that, with respect to the presence of dysplasia, interobserver agreement was moderate, with greater agreement for high-grade dysplasia. In addition, when endoscopic data and histological data were combined, dysplasia-associated lesions or masses were more common than adenoma-like masses. Therefore, the authors proposed using immunohistochemistry to increase the capacity for detecting these lesions, as well as a review of samples by a pathologist specializing in digestive diseases[49].

NEW ENDOSCOPIC TECHNIQUES

Full-spectrum endoscopy

Full-spectrum endoscopy (FUSE) comprises 2 Lateral cameras in addition to the traditional front camera, and this configuration makes it possible to see behind the folds and blind spots, thus providing a panoramic view.

Leong *et al*[50] performed a prospective, randomized, crossover tandem colonoscopy study of 52 patients to compare standard forward-viewing colonoscopy (FVC) with FUSE for the detection of dysplasia in patients with IBD. FUSE revealed significantly more dysplastic lesions than FVC, although the withdrawal time was significantly greater. Targeted biopsy revealed significantly more dysplastic lesions than random biopsy ($P < 0.0001$).

Autofluorescence imaging

Autofluorescence imaging (AFI) is based on the detection of natural tissue fluorescence emitted by endogenous molecules (fluorophores) such as collagen, flavins, and porphyrins. After excitation by a short-wavelength light source, these fluorophores emit light of longer wavelengths (fluorescence). The overall fluorescence emission differs between the various tissue types owing to the corresponding differences in fluorophore concentration, metabolic state, and/or spatial distribution. These colour differences in fluorescence emission can be captured in real time during endoscopy and used for the detection or characterization of lesions[51]. A multicentre international prospective randomized controlled trial compared images taken with AFI with chromoendoscopy for the detection of dysplasia in 210 patients with UC. The relative detection rate for dysplasia had to be greater than 0.67 (based on an 80% confidence interval) to justify a subsequent noninferiority trial. Dysplasia was detected in 20 patients (19%) using chromoendoscopy and in 13 (12%) using AFI. Therefore, AFI proved to be inferior to chromoendoscopy, and the criteria for performing a large-scale noninferiority trial were not fulfilled. The authors concluded that AFI should not be investigated as an alternative approach for monitoring the presence of dysplasia[52].

Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is a new method used for obtaining *in vivo* images of abnormalities of the mucosa at the subcellular level. The technique uses intravenous fluorescent agents, contrast, and a specialized probe that can be passed through the working channel of an endoscope, thus enabling real-time 1000-fold magnification of the mucosa of the colon. CLE has been shown to help differentiate among neoplasms, solitary adenomas, and benign regenerative changes (which can be seen in much the same way as dysplasia), with 97.8% accuracy[53].

Pilot trials have been performed with variations of this technique. One study evaluated the potential role of CLE combined with a fluorescein-labelled peptide to stain and detect dysplasia associated with UC. A heptapeptide derived from phages with marked affinity for dysplastic tissue was synthetized and labelled with fluorescein (VRPMPLQ peptide). The study included 9 patients who underwent the resection of 11 Lesions. The different affinities of fluorescein for nondysplastic tissue, inflammatory polyps, and dysplastic tissue enabled better characterization. The authors concluded that VRPMPLQ is a promising approach for the detection of dysplasia in patients with IBD. More *in vivo* studies in larger populations are required to evaluate the effective contribution of this molecular probe in the management of lesions detected during the surveillance of patients with UC[54]. However, the results of these studies should be confirmed in larger series. CLE is currently restricted to

research.

Endocytoscopy

Endocytoscopy is a novel ultra-high magnification endoscopic technique designed to provide excellent *in vivo* assessment of lesions found in the gastrointestinal tract. When used with intraprocedural stains, endocytoscopy enables microscopic visualization of the gastrointestinal mucosal surface[55,56].

Nevertheless, the application of endocytoscopy in the diagnosis of colitis-associated CRC has received little attention. A recently published case report revealed that endocytoscopy can be used to obtain information on the nuclei and lumen of the glands in colitis-associated CRC. Furthermore, colitis-associated CRC is often difficult to diagnose owing to the effects of inflammation, and the use of endocytoscopy can reduce the frequency of unnecessary biopsies[57].

DEVICES FOR IMPROVING ENDOSCOPIC VISION

Endocuff Vision (ARC Medical Design Ltd) is a distal colonoscopic accessory with smooth projections in the form of fingers that aims to flatten mucosal folds, and it has been shown to improve the rate of the detection of adenomas in patients with IBD undergoing screening for CRC[58]. The device is not currently recommended in patients with severe colitis because of concerns over mucosal injury[59], although it has not been previously studied in clinically active patients with IBD undergoing surveillance colonoscopy for the detection of dysplasia. A study of 25 patients evaluated the safety and viability of Endocuff Vision–assisted high-definition chromoendoscopy in patients with clinically active UC undergoing surveillance for dysplasia. The authors concluded that the technique is feasible and safe in patients with UC undergoing surveillance colonoscopy to rule out dysplasia[60]. Other studies reported that Endocuff could facilitate polypectomy, especially in the flexible folds of the sigmoid colon, thus enabling greater stabilization of the endoscope when facing the polyp[61]. In the future, it will be necessary to determine whether these devices can increase the rate of detection of polyps and facilitate their extraction without increasing the rate of complications in patients with UC.

COLON CAPSULE ENDOSCOPY

A priori, capsule endoscopy is an attractive option for screening CRC in patients with IBD because it is noninvasive and has proven useful in certain situations, namely, after incomplete colonoscopy, when the patient refuses colonoscopy, or when sedatives are contraindicated.

Regarding its yield, second-generation capsule endoscopy (CE-2) is almost at the same level as colonoscopy, with 80%–95% sensitivity for the detection of polyps ≥ 6 mm. The limitations of CE-2 in CRC are that it cannot be used for biopsy, for obtaining samples of the intestinal mucosa, or for resecting any lesions detected[62].

This is a field for future research, which will undoubtedly provide huge surprises in the coming years. Prototypes with steerable and self-propelling technology have already been designed[63].

COMPUTER-AIDED DETECTION

Nonblinded trials have shown that colonoscopy with computer-aided detection (CADe) improves the detection of polyps and adenomas in the colon by providing visual alarms during the procedure[64]. The technique was developed in China in a randomized, double-blind clinical trial aimed at evaluating the efficacy of a CADe system. The study excluded patients with IBD. The rate of the detection of adenomas was significantly greater in the CADe group than in the control group[65]. In the future, it will be necessary to evaluate whether computational devices that facilitate detection are valid for patients with IBD.

COSTS ASSOCIATED WITH SCREENING

Few studies have evaluated the costs—and more especially the cost-effectiveness—of screening in patients with UC. One of the first studies to do so was published by Provenzale *et al*[66] in 1998, who analysed the cost-effectiveness of CRC screening programmes and found a favourable association with screening. However, endoscopic technology has changed considerably since then.

The study by Konijeti *et al*[67] in 2014 evaluated 3 strategies with different surveillance intervals—chromoendoscopy with targeted biopsy, white-light endoscopy with random biopsy, and no surveillance—taking into account the financial costs and the characteristics of the United States health system. However, compared with no surveillance, chromoendoscopy was only cost-effective at intervals of at least 7 years, with an incremental cost-effectiveness of $77176. Chromoendoscopy was the most cost-effective strategy, with a sensitivity > 0.23 for the detection of dysplasia and a cost < $2200, regardless of the sensitivity of WLE for the detection of dysplasia. The estimated population lifetime risk of developing CRC ranged from 2.5% (annual chromoendoscopy) to 5.9% (chromoendoscopy every 10 years).

This aspect should be evaluated in the different health systems, given the large potential differences in the cost of screening programmes between countries.

CHEMOPREVENTION

Cancer chemoprevention involves the chronic administration of a synthetic, natural, or biological agent to reduce or delay the occurrence of malignancy[68]. In addition, the agent administered must be effective, safe, acceptable to patients, and inexpensive.

Chronic inflammation of the mucosa in patients with UC is the main risk factor for the development of CRC[69].

No medical treatment has proven able to sufficiently prevent dysplasia or CRC and thus obviate the need for colonoscopic surveillance in UC[23].

Chemoprevention is considerably controversial owing to the diversity of studies and varying reports on adherence to treatment, and available evidence supports the role of 5-ASA as chemopreventive therapy[70]. Immunomodulators could play a role owing to their control of mucosal inflammation, at least in a subgroup of patients[71]. The control of inflammation achieved with anti-TNFa agents would probably reduce the risk of CRC in patients with UC. Even in more recent observational studies, the number of patients exposed to tumour necrosis factor-alpha inhibitor agents is too low to adequately evaluate the potential chemopreventive effects of these agents[71]. In UC and concomitant PSC, low-dose ursodeoxycholic acid could reduce the risk of CRC, although evidence for this hypothesis is weak[72,73].

While much remains to be done in the field of the chemoprevention of CRC in patients with UC, the main barrier in the coming years will be the difficulty in performing clinical trials that provide more robust evidence.

CONCLUSION

Despite the advances made in the field, the following areas warrant further study: (1) The tailoring of surveillance intervals to the individual patient; (2) Continuous updating of recommendations based on the best available evidence; (3) Greater adherence by physicians to the recommendations of scientific societies; (4) The development of noninvasive biomarkers that could support or act as a screening approach before endoscopy; and (5) Efforts to reduce barriers to surveillance, including safe extension of intervals in patients with a lower risk.

The challenge in the coming years will be to match the individual patient with the best option for endoscopic surveillance. We must bear in mind the large number of newly diagnosed patients, in addition to those currently under surveillance and the fact that they are ageing, together with the increasing price of new technology and surveillance modalities. The maintenance of an adequate and efficient physician-patient relationship in decision making will be the greatest challenge we have to face in the coming years.

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Viral hepatitis: Innovations and expectations

Simona Leoni, Alberto Casabianca, Benedetta Biagioni, Ilaria Serio

ORCID number: Simona Leoni 0000-0001-8825-9698; Alberto Casabianca 0000-0003-2482-9422; Benedetta Biagioni 0000-0002-5152-0626; Ilaria Serio 0000-0002-6676-4148.

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Simona Leoni, Alberto Casabianca, Benedetta Biagioni, Ilaria Serio, Division of Internal Medicine, Hepatobiliary and Immunoallergic Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna 40138, Italy

Corresponding author: Simona Leoni, MD, PhD, Doctor, Division of Internal Medicine, Hepatobiliary and Immunoallergic Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Via Albertoni 15, Bologna 40138, Italy. simona.leoni@aosp.bo.it

Abstract

Viral hepatitis is a significant health problem worldwide, associated with morbidity and mortality. Hepatitis B, C, D, and occasionally E viruses (HBV, HCV, HDV, and HEV) can evolve in chronic infections, whereas hepatitis A virus (HAV) frequently produces acute self-limiting hepatitis. In the last years, different studies have been performed to introduce new antiviral therapies. The most important goal in the treatment of viral hepatitis is to avoid chronic liver disease and complications. This review analyzes currently available therapies, in particular for viruses associated with chronic liver disease. The focus is especially on HBV and HCV therapies, investigating new drugs already introduced in clinical practice and clinical trials. We also describe new entry inhibitors, developed for the treatment of chronic HDV and HBV and currently available treatments for HEV. The last drugs introduced have shown important efficacy in HCV, with achievable target HCV elimination by 2030. Concurrently, renewed interest in curative HBV therapies has been registered; current nucleotide/nucleoside analogs positively impact liver-related complications, ensuring high safety and tolerability. Novel approaches to HBV cure are based on new antivirals, targeting different steps of the HBV life cycle and immune modulators. The improved knowledge of the HDV life cycle has facilitated the development of some direct-acting agents, as bulevirtide, the first drug conditionally approved in Europe for HDV associated compensated liver disease. Further studies are required to identify a new therapeutic approach in hepatitis E, especially in immunosuppressed patients.

Key Words: Viral hepatitis; Chronic liver disease; Treatments; Antiviral; Immunotherapy; Vaccination

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Core Tip: Viral hepatitis is a known worldwide health problem, with a risk of evolution in chronic liver disease. Novel therapies have shown increased efficacy in curing the hepatitis C virus (HCV), with the goal of HCV elimination by 2030. New concurrent interest in hepatitis B virus (HBV) curative therapies has been recently registered: New antivirals targeting different steps of HBV life cycle and immune modulators. In hepatitis D, the improved knowledge of the life cycle has facilitated the development of some direct-acting agents, more effective than interferon-based therapies. New studies are required to improve the treatment of hepatitis E.

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INTRODUCTION

Viral hepatitis represents a health problem that affects millions of people worldwide and is associated with high mortality. Except for hepatitis A virus (HAV), all hepatotropic viruses, including hepatitis B, C, D, and E viruses (HBV, HCV, HDV, and HEV), can produce chronic infections, whereas HAV causes acute self-limiting hepatitis that normally resolves spontaneously. In this review, we provide a brief outline of currently available therapies for major hepatotropic viruses associated with chronic liver disease. We focus especially on therapies that halt HBV progression and achieve a definitive HCV cure, investigating new drug clinical trials. We also describe bulevirtide, an entry inhibitor, developed for the treatment of chronic HDV and HBV infections and approved in the European Union for the therapy of chronic HDV infection in patients affected by liver disease, without decompensation. We describe currently available treatments for HEV with their limitations, pointing out the emergent need for novel antivirals not only safe but above all effective particularly for patients not eligible for interferon (IFN)-based regimes.

HEPATITIS B

HBV infection is the main cause of chronic liver disease in the world, affecting 257 million people globally, and is responsible for about 54% of the cases of hepatocellular carcinoma (HCC)[1].

The current goal for HBV treatment is to suppress viral replication, halt disease progression, and prevent cirrhosis development, liver failure, and HCC. The approved agents for the treatment of chronic HBV infection are not curative and can be classified into two categories: Pegylated IFN (peg-IFN) and nucleotide or nucleoside analogs (NAs)[2].

In particular, there are eight approved drugs: IFN and peg-IFN, lamivudine, adefovir, telbivudine, entecavir, tenofovir disoproxilfumarate (TDF), and tenofovir alafenamide fumarate (TAF). Besifovir dipivoxil is only approved in Korea. Their action is limited to suppress HBV replication and decelerate progression to cirrhosis and HCC.

Current and future aims of treatment

The current aim of antiviral treatment is the induction of two different responses: Virologic and biochemical[3].

Virologic response: A virologic response during NA therapy is defined as undetectable HBV-DNA. In the course of IFN treatment, a virologic response is obtained if serum HBV-DNA level falls below 2000 IU/mL, 6 mo after the start of treatment and at the end of treatment.

Biochemical response: A biochemical response is obtained when alanine aminotransferase falls into the normal range.

Functional cure: Corresponding to a durable hepatitis B surface antigen (HBsAg) loss, with or without seroconversion, is achieved rarely in the natural history of chronic hepatitis B: In only 10%-20% of Caucasian patients and less than 5% Asian patients[4]. HBsAg seroclearance correlates with a reduced risk of HCC, therefore it represents an advisable goal of treatment. The functional cure aspires to sterilize cells neither from integrated HBV-DNA nor from covalently closed circular DNA (cccDNA), a persistent form of the HBV genome in infected hepatocytes.

Complete cure: Complete cure is a functional cure with the elimination of cccDNA, which acts as a potential reservoir for reactivation. Complete HBV elimination is difficult, due to the existence of intrahepatic cccDNA and its ability to self-replenish: cccDNA serves as a template for the transcription of pre-genomic RNA, allowing the production of viral DNA and proteins even without detectable HBV-DNA or HBsAg [5,6]. A complete HBV cure consists of a full HBV eradication through the removal of all viral elements from an infected patient. To achieve this goal, combination therapy is probably required[7]. Treatments currently available, peg-IFN and NAs, have no direct effect on cccDNA that persists in the hepatocyte nucleus.

An additional obstacle to HBV elimination is the viral DNA integration into the host genome, sufficiently intact to support the translation of viral protein[8]. Integrated HBV-DNA can be a source of circulating HBsAg; definitive clearance of HBV would require the removal of hepatocytes that harbor this DNA.

The persistence of HBV in the liver of patients who have recovered from acute HBV infection can explain the risk of HBV reactivation in the course of potent immunosuppressive therapy and the potential transmission of HBV infection when these livers are transplanted into seronegative recipients[9].

An additional limitation to HBV elimination is impaired innate and adaptive immune responses in HBV patients. Chronic HBV patients fail to unleash efficient immune responses to HBV if cytotoxic T cells are hypo-responsive to HBV itself. For a complete cure, the restoration of suppressed host immunity is required[10].

New biomarkers in diagnosis and management of HBV infection

To diagnose acute and chronic HBV infections, some tests are routinely used. The identification of HBV presence and activity is essential in the diagnostic and therapeutic algorithm of infected patients. In clinical practice, there are routinely used assays to detect and measure serum levels of HbsAg, HbeAg, anti-HBs, and HBV DNA. Some of them are strong risk predictors of the development of HCC[11].

New diagnostic tools are needed to assess the efficacy of therapy and monitor with precision the response. The most interesting includes tests to measure HBV RNA and hepatitis B core-related antigen (HBcrAg). Levels of serum RNA indicate the presence of cccDNA transcriptional activity and might be used to monitor response to treatment and identify patients eligible for the safe discontinuation of NA. Also, HBcrAg might be used as a marker of cccDNA and its quantification could help to monitor response to current therapies[12]. New biochemical tools are in development to improve the diagnostic approach to virus activity and reservoir.

Available treatments: Pros and cons

Peg-IFN has lower antiviral activity but a higher rate of HBeAg and HBsAg loss than NAs, thanks to its immunomodulatory effect. It is administered as subcutaneous injections, which is a clear drawback, once weekly. It amounts to multiple adverse effects. And its use is strictly prohibited in patients with decompensated cirrhosis and autoimmune and psychiatric illnesses. Caution is mandatory in patients with compensated cirrhosis because it may precipitate hepatic decompensation. A 1-year course of pegylated IFN results in a rate of HBeAg seroconversion and HBsAg loss of 30% and 3%, respectively, in patients who are HBeAg-positive. Undetectable HBV-DNA is achieved in 25% of cases. The response is durable and rates of HBeAg and HBsAg loss increase after the end of treatment (to about 8%). The finite duration of treatment (48-52 wk) is a strong point[13].

NAs are administered orally, have irrelevant adverse effects, and can be safely administered in patients with decompensated cirrhosis or acute liver failure[14]. Their main drawback is the need for long-term treatment in the majority of the patients.

Among NAs, ETV, TDF, and TAF are normally preferred; they boast high antiviral activity and barriers to resistance. After 1 year of treatment of HBeAg positive patients, NAs allow to get HBeAg seroconversion and HBsAg loss, respectively, in 10%-21% and < 1%-3%. Rates of undetectable HBV-DNA (61%-76%) are higher than those achieved by IFNs. HBeAg negative patients treated for a year with pegylated

IFN, achieve a higher rate of HBsAg loss (4% *vs* 1%) than the same duration of ETV, TDF, or TAF, despite a lower rate of HBV-DNA clearance (63% *vs* 94%). TAF is a prodrug of TDF, a nucleotide analog that inhibits reverse transcription of the human immunodeficiency virus (HIV) and HBV[15]. It has similar antiviral activity as TDF but its superior plasma stability and more effective active metabolites, allow the use of lower doses with similar antiviral activity and less systemic exposure. It has, therefore, a smaller damaging impact on glomerular filtration rate and bone mineral density.

What's in store for the future? Antivirals

Following the mirage of a sterilizing cure able to eliminate both cccDNA and integrated HBV-DNA, new approaches are being developed for the treatment of HBV.

Here we describe new drugs for HBV, including drugs that target different steps of the HBV life cycle and immune modulators. Some of them are under phase 2 trials.

Entry inhibitors: Some novel drugs aim to block HBV entry into hepatocytes that may protect hepatocytes not yet infected or antagonize *de novo* infections. The sodium taurocholate co-transporting polypeptide (NTCP) is the entry receptor for HBV/HDV into hepatocytes. Bulevirtide (Myrcludex B) is a peptide that mimics the NTCP-binding domain of HBV, blocking HBV and HDV entry into naïve hepatocytes[16].

Bulevirtide is approved in the European Union for the treatment of chronic HDV infection in compensated liver disease.

A multicenter, open-label phase 2 clinical trial (MYR203) assessed the efficacy of myrcludex B in combination with peg-IFNα2a in HDV/HBV co-infected patients. Sixty co-infected patients were randomized to receive peg-IFN or peg-IFN plus bulevirtide, or bulevirtide for 48 wk. Among the 60 patients, 27% of them in the peg-IFN plus bulevirtide group achieved HBsAg loss at 48 wk of treatment[17].

NTCP is a transporter of conjugated bile salts from the plasma into hepatocytes. Consequently, adverse events such as elevated plasma bile acids were reported in a dose-dependent manner but without clinically relevant effects (such as cholestasis and consequent pruritus or steatorrhea)[18].

Most of these studies are focused on HBV/HDV co-infection, but we expect the result of a new trial in chronic hepatitis B mono-infection.

Capsid assembly modulators: Inhibiting nucleocapsid formation is a promising strategy for HBV therapy. Nucleocapsid contains the viral DNA needed for replication. Two core protein allosteric modulators (CpAM-capsid assembly modulators) exist: Class I CpAMs lead to the formation of misassembled capsids. Class II CpAMs accelerate capsid assembly and form morphologically normal capsids that are empty.

NVR 3–778, first-in-class CpAM, has been evaluated in different studies. In a phase 1 study of HBeAg-positive patients with chronic infection, NVR 3-778 was well tolerated[19]. The largest drop in HBV-DNA levels was achieved in the cohort treated with NVR 3-778 in combination with pegIFN.

GLS4 is an inhibitor of HBV capsid assembly that induces the formation of aberrant nucleocapsid structures. A randomized, open-label phase 1b study examined the efficacy of GLS4 (at different doses) in combination with ritonavir for treating chronic HBV infection compared with entecavir alone. GLS4 120 mg administrated for 28 d, resulted in a reduction in serum HBV-DNA and HBV pre-genomic RNA (pgRNA) levels without major side effects[20].

RO7049389 disrupts HBV nucleocapsid assembly and induces the depletion of core proteins, thereby effectively inhibiting HBV replication. A multicenter, ongoing phase 1 study is investigating the efficacy of single and multiple doses of RO7049389 in healthy volunteers and chronic HBV participants. RO7049389 administration to HBV patients at different dosages, for 28 d, achieved a drop of HBV-DNA and RNA, in both HBeAg positive and negative patients with HBV-DNA levels lower than the lower limit of quantification in 81.3% of them[21].

JNJ-6379 (JNJ-56136379) is a class II CpAM that interferes with capsid assembly and inhibits *de novo* formation of cccDNA. A phase II study is ongoing. JNJ-6379 administrated for 4 wk to treatment-naïve patients with CHB, showed potent antiviral activity: HBV-DNA and HBV-RNA decreased from baseline in all patients. On day 29, 32% of 41 patients had levels of HBV-DNA below the lower limit of quantification[22]. The most interesting drop of HBV-DNA and RNA was found in the Asian cohort treated at a dose of 75 mg/d. After discontinuation of treatment, the viral load returned to baseline levels[23]. JNJ-6379 did not interfere with HBsAg or HBeAg.

ABI-H0731 (vebicorvir) is a potent and selective class II CpAM. In the 101B study, ABI-H0731 was administrated to HBeAg-positive patients for 28 d and obtained a

dose-dependent HBV-DNA reduction (maximum decline of 4.0 $\log_{10}$ IU/mL) with a parallel HBV RNA decline. No changes in HBsAg, HBeAg, or HBV core-related antigen were reported[24].

JNJ-64530440 and QL-007**,** two assembly modulators, and ABI-H2158, a core protein binding, are further interesting objects of study.

We need to further studies to establish whether CpAMs can eliminate HBsAg, HBeAg, and/or cccDNA but the combination strategy seems to be the better one.

Post-transcriptional inhibitors: Synthetic small interfering RNAs (iRNA) or silencing RNAs, are a class of non-coding RNAs that recognize complementary viral mRNA and pgRNA, inducing mRNA degradation after transcription and preventing translation [25]. In the contest of HBV infection, small iRNAs bind HBV mRNA in hepatocytes and induce its degradation. Their target is all RNA transcripts, derived from cccDNA [26].

ARC-520 was the first iRNA therapeutic targeting HBV. A single-dose phase 2 study showed that HBsAg was reduced more in HBeAg positive patients than in HBeAg negative or treatment-experienced patients[8]. To explain these findings, it should be recalled not only that HBsAg production mostly depends on integrated HBV-DNA, which is the main source in HBeAg negative and NA-experienced patients but also that ARC-520 is not able to target mRNA produced by integrated HBV-DNA.

Two randomized multicenter studies evaluated the HBsAg decrease after multiple doses of ARC-520 compared to placebo, in NA–experienced HBeAg negative or positive patients[27]. A high dose of ARC-520 (2 mg/kg) reduced HBsAg. The absolute reduction was larger in the HBeAg positive cohort, confirming that a significant proportion of HBsAg, in HBeAg negative patients, could derive from integrated HBV sequences. Ideal iRNA should target all viral transcripts. A second-generation iRNA targeting all HBV transcripts, ARC-521, reduces HBsAg and viral load as demonstrated in a phase I trial (NCT02797522). Both ARC-520 and ARC-521 studies were stopped for the lethal toxicity of specific delivery vehicles in non-human primates. A modified iRNA, JNJ-3989 (formerly ARO-HBV), is under investigation (NCT03365947). The HBsAg decline is strong in treatment naïve and experienced patients regardless of HBeAg status due to its ability to silence mRNA from cccDNA and host integrated viral DNA. ARB-1467 was tested in a phase II study, showing a consistent decline of HBsAg in 63.6% of patients (NCT02631096). An ongoing study is evaluating the efficacy of VIR-2218 in infected volunteers[28]. Preliminary results are promising to show a decline in HBsAg, but we are waiting for official data.

Inhibitors of HBsAg release: HbsAg proteins are not only incorporated into the viral envelope but also stored in non-infectious subviral envelope particles (SVP) produced by HBV, independently of viral replication. These particles, without capsid or genome, account for an important source of HBsAg in the blood and induce immune exhaustion against HBsAg. To eliminate spherical SVP is a major goal of a functional cure. Nucleic acid polymers (NAPs), such as REP 2139, target the assembly/secretion of SVPs. An open-label, phase 2 study tested the effectiveness of REP 2139 or REP 2165 combined with TDF and peg-IFN in HBeAg-negative CHB patients. The addition of NAPs to TDF and peg-IFN, without reducing the tolerability, significantly increased rates of HBsAg loss and HBsAg seroconversion during therapy[29]. After 12 mo of treatment, HBsAg levels were very low in more than half of the patients and after a temporal interval of 1 year, virologic control persisted in about half of them.

Neutralization: Lenvervimab is a recombinant human monoclonal IgG1-type anti-S antibody that binds HBsAg, inhibiting viral penetration into hepatocytes and reducing HBsAg levels. The neutralization of virions or HBsAg is obtained by immune complexes. The HBV neutralizing activity of lenvervimab had previously been demonstrated in a chimpanzee animal model[30].

In a prospective, open-label phase I trial, a single injection or 4 weekly doses of lenvervimab were administered (doses from 80.000 to 240.000 IU) to HBeAg-positive patients, reducing levels of HBsAg to below undetectable levels for up to 1 mo[31].

Inhibitors of cccDNA: As mentioned above, viral transcription requires a template, consisting of cccDNA. Curative treatment for HBV infection would require a disablement of cccDNA and several mechanisms have been studied to eradicate or silence cccDNA expression. The possibility of using sequence-specific RNA-guided nucleases (RGNs) and proteins has been explored. Among RGNs, the best present results are linked to transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) systems[32-34].

Nucleases cleave sequences in the HBV genome, resulting in mutated cccDNA, which is, in turn, transcribed into mutated viral proteins, not adequate to viral replication. Preclinical experiments showed that the CRISPR/Cas9 system, the most efficient method for gene inactivation, elicits inactivation of cccDNA, through mutations and deletions, but the application of this technology in a clinical setting requires further studies. For the clinical application, several challenging issues must be solved, first of all, the risk of genome instability.

Table 1 summarizes the main molecules to treat hepatitis B with the synthesis of the mechanism of action.

What's in store for the future? Immunotherapy

The progression to chronic HBV infection seems to be favored by weak immune responses and labile innate immune responses. It has been demonstrated that, in "recovered" patients, the early HBsAg clearance is fostered by activation of different cellular receptors [Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)] that allow the production of antiviral cytokines (IFN-α primarily), and, activation of natural killer (NK) cells. Activated innate immunity also boosts the adaptive immune system, leading to the maturation of B- and T-cell clones that target infected hepatocytes.

The progression to chronic HBV infection is determined by dysfunctional NK cells and plasmacytoid dendritic cells (pDCs). High levels of HBsAg or HBeAg not only block maturation of antigen-presenting cells such as DCs, causing a tolerogenic environment, but also deregulate the production of IFN and other cytokines by NK cells, NK T cells, and Kupffer cells. Prolonged exposure to viral antigens could be associated with functionally impaired immune response against the virus. Furthermore, HBV-specific cytotoxic T cell response, essential for clearance of infected hepatocytes, is inadequate with a poor cytotoxic activity and impaired cytokine production. This phenomenon has been described as "T cell immune exhaustion" and is responsible for HBV persistence. Several studies have reported overexpression of programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) in CD4+ or CD8+ T cells during chronic HBV infection.

Among compounds highly active against innate immunity, some immune stimulators (such as inarigivir) and TLR agonists (such as TLR7 and TLR8) have been studied. Among immunologic agents with activity against adaptive immunity, we must remember therapeutic vaccines (*e.g.*, GS-4774) and anti-PD-1 antibodies (*e.g.*, nivolumab) that are objects of ongoing studies.

TLR agonists: TLRs constitute the first line sensors of microorganisms and activate the production of mediators including IFNs and cytokines. TLR agonists promote up-regulation of type I IFN and cytokines, stimulating innate immunity response directed against the virus. The activation of TLR mediated pathways leads to the suppression of HBV replication. As an agonist of TLR-7 involved in IFN production, GS-9620 was tested in a human hepatocyte cell line infected with HBV; it showed suppression of HBV replication but no reduction in cccDNA levels. In woodchucks and chimpanzees, the TLR-7 agonist showed similar results. When used in humans with HBV-DNA suppression in the course of NA therapy, TLR-7 agonist (vesatolimod GS-9620) did not obtain any effect on HBsAg, HBeAg, and HBV-RNA levels despite inducing an HBV specific immune response. Another agonist for TLR-8 (Selgantolimod GS-9688) was investigated in patients with CHD and chronic suppression of the viral load, showing no significant impact on HBsAg, HBV-DNA, or RNA. Now this study is in phase II [35]. Several other TLR-7 and TLR-8 and 9 agonists are now entering clinical trials.

Retinoic acid-inducible gene-1 agonists: Type III IFNs are produced in human hepatocytes against HBV, through RIG-I. Viral RNA activates this intracytoplasmic receptor, leading to the IFN response that is essential for antiviral immunity[36]. Inarigivir is an immune modulator with an antiviral effect. It acts as a RIG-I agonist, inducing type I and type III IFN production, thus boosting immune response. In the ACHIEVE study, 80 treatment naïve non-cirrhotic patients with chronic HBV infection were enrolled. They received, through a random sampling, different doses of inarigivir or placebo for 3 mo followed by tenofovir for 3 mo. Both HBeAg-positive and negative patients achieved HBV-DNA and RNA reductions in a dose-dependent manner, and an HBsAg decline of > 0.5 $\log_{10}$ at 12 or 24 wk was found in more than 20% of patients.

Immune checkpoint inhibitors: Apoptosis is a fundamental mechanism at the basis of HBV-specific CD8 T cell depletion. Interruption of this mechanism restores CD8 T-cell responses against HBV. In the setting of chronic HBV infection, inhibitory receptors, like PD-1 and programmed death-ligand 1 (PD-L1) are overexpressed on exhausted T

Table 1 New antiviral drugs for hepatitis B

	Molecule(s)	Mechanism of action	Ref.
Entry inhibitors	Bulevirtide (Myrcludex B)	Block HBV entry into hepatocytes to protect cells not yet infected or antagonize *de novo* infection	[16, 17]
Capsid assembly modulators	NVR 3-788; GLS4; RO7049389; JNJ-6379; JNJ-0440; ABI-H0731	Inhibition of nucleocapsid formation	[19-24]
Post-transcriptional inhibitors	ARC-520; ARC-521; ARB-1467	Induction mRNA degradation after transcription and translation prevention	[27, 28]
Inhibitors of HBsAg release	REP 2165; Lenvervimab	Removal of subviral envelope particle. Neutralization activity	[29-31]
Inhibitors of cccDNA	CRISP-Cas system	Disablement of cccDNA	[32-34]

HBsAg: Hepatitis B surface antigen; cccDNA: Covalently closed circular DNA; HBV: Hepatitis B virus.

cells, limiting the effector function[37]. The block of the PD-1/PD-L1 pathway seems to enhance their function. In a few words, checkpoint inhibitors may restore T cell dysfunction.

In a recent phase I pilot study, the efficacy of the PD-1 inhibitor nivolumab was investigated. Twenty-four NA-suppressed anti-HBe positive patients received nivolumab at two different dosages, combined or not with the therapeutic vaccine GS4774. Patients that received higher doses had a noticeable decline in HBsAg levels and one of the patients receiving both nivolumab and the therapeutic vaccine GS4774 lost HBsAg[38]. Exhausted CD8+ T cells present also co-inhibitory molecules such as CTLA-4 and CD244/2B4 whose blockade, *in vitro* studies, seems to achieve restoration of immune function.

Stimulator of interferon genes agonists: The use of synthetic agonists for activation of the stimulator of interferon genes (STING) seems to stimulate the production of cytokines, especially IFNs. In mouse models, intraperitoneal infusion of a STING agonist stimulated the expression of IFN genes and mitigated HBV replication in mouse hepatocytes[39]. Such agonists may be exploited for the development of novel anti-HBV strategies.

Problem of antiviral resistance in chronic hepatitis B

Nucleoside and nucleotide analogs inhibit the viral DNA polymerase, causing an early stop of HBV replication. Drug-resistant strains of HBV have mutations in the viral polymerase gene. Resistance mutations alter the interaction and the inhibition of the drug on the viral polymerase. These mutants persist even after the end of treatment and determine cross-resistance to the next drug[40]. To prevent resistance development, guidelines recommend, as the first approach, the use of NAs with a high barrier to resistance. Sequential monotherapies with a low barrier to resistance should be avoided. In all cases of treatment failure, it is essential to precociously identify possible resistance mutations without forgetting to always check compliance to NAs[2].

What's in store for the future? Therapeutic vaccination

The therapeutic vaccine has shown encouraging results in animal models; non-similar results were demonstrated in humans for both vaccines, with adjuvant or with DNA. They are developed to stimulate the host immune response to suppress HBV replication and foster HBsAg loss. Therapeutic vaccination, alone or associated with oral antiviral therapy, is not sufficient to restore HBV immunity in chronically infected patients. Combination strategy failure was demonstrated by some studies, as the randomized study of Vandepapelière *et al*[41]: The difference between the HBe seroconversion rate obtained by the co-administration of vaccine and lamivudine (18.8%) or by lamivudine alone (16.1%) was not significant[41].

Previous vaccines only targeted HBsAg, but the new ones, under development, are against multiple HBV proteins and novel adjuvants. GS-4774 and TG-1050 are precisely the results of approaches based on multiple HBV proteins.

GS-4774 is a vaccine that uses recombinant *Saccharomyces cerevisiae* yeast to express surface, core, and X proteins. Virally suppressed patients, on treatment with oral NAs, were randomized to continue antiviral therapy alone or associated with different yeast

units of GS-4774. GS-4774 did not determine significant reductions in HBsAg levels. Five HBeAg-positive patients treated with GS-4774 showed HBeAg loss while none in the control group showed seroconversion to anti-HBe. Increased production of IFN-α, TNF-α, and IL2 by CD8+ T cells was documented[42].

TG1050 is an adenovirus-based vaccine that encodes a fusion protein composed of HBV polymerase and domains of HBcAg and HBsAg. This is a T-cell-inducing vaccine and has demonstrated antiviral effects in mice. In CHB patients treated with NAs, TG1050 is safe and effective in inducing HBV-specific cellular immune response. It induced functional IFN-γ producing cells and despite that serum HBsAg did not decrease significantly following TG1050, a robust decrease of HBcAg was shown in some patients[43].

Despite unsatisfactory results, it is now clear that a therapeutic vaccine could effectively achieve a functional cure only if associated with NAs or immunomodulatory therapies.

HEPATITIS D

HDV is a highly pathogenic virus that causes acute, fulminant, and chronic hepatitis, causing cirrhosis in more than 70% of cases. HDV has been defined as a defective virus because it needs the helper function of HBV for its transmission. The two possible scenarios are simultaneous infection of HDV and HBV (coinfection) that leads to chronic hepatitis D in < 5%, or HDV superinfection in chronic hepatitis B patients (superinfection); in this case, chronic hepatitis occurs in up to 90% of patients[44,45].

Permanent HDV-RNA suppression is associated with improved clinical outcomes. The treatment for chronic HDV infection with IFN therapy achieves a virologic response rate of about 17%-47%[46,47]. This translates into a rare curative outcome. Moreover, late viral relapses frequently occur in patients with chronic HDV hepatitis who have achieved sustained virological response (SVR). The treatment of choice in patients with HDV and HBV infection with compensated liver disease is peg-IFNa for 48 wk. The serious side effects (influenza-like illness, alopecia, leucopenia, thrombocytopenia, and emotional lability) make IFN a drug with huge limits.

NA treatment is also recommended when HBV-DNA levels are constantly less than 2.000 IU/mL and in patients with advanced liver disease[2].

In the last years, many efforts have been made to identify new therapeutic targets for HDV treatment. As mentioned above, NTCP is an important receptor for HBV to enter hepatocytes. HDV virions also attack the viral receptor NTCP before membrane fusion and release of the ribonucleoprotein into the cytoplasm. The entry of HDV into new cells can be blocked by bulevirtide (Myrcludex B) according to its action on a specific receptor NTCP. Bulevirtide is a chemically synthesized peptide composed of amino acids derived from large HBV surface proteins. Bulevirtide was recently approved in the European Union for treatment of chronic hepatitis D in HDV-RNA positive adult patients when liver disease is compensated. Bulevirtide blocks the virus entry into the cells and limits the HDV replication capacity. As mentioned above, two main studies showed that bulevirtide is effective at clearing HDV-RNA from the blood. In the first study, the MYR202 study, 55 out of 90 patients who received bulevirtide and TDF lost HDV-RNA after 6 mo, compared with 1 out of 28 patients treated with TDF alone. The combination treatment was associated with a significant normalization in alanine aminotransferase and an interesting improvement of liver function, as compared to patients who received TDF alone[48].

Similar results were seen in the second study, the MYR203, where patients were randomized to bulevirtide plus peg-INF alpha-2a, bulevirtide alone, and PEG-IFN alpha-2a alone for 48 wk[17]. At 48 wk, HDV-RNA was undetectable in 80% of patients treated with bulevertide 2 mg plus peg-INF alpha-2a while lower rates were obtained in the other two groups. Final results at week 72 confirmed the effectiveness of the association strategy, showing undetectable HDV-RNA in 53.3% of cases *vs* 0% in the IFN group[49].

A fundamental step in the virus assembly process consists of the modification (prenylation) of a specific HDV protein. This protein is the large delta antigen (HDAg). Prevention of prenylation blocks the formation of viral particles or more precisely, the assembly of mature HDV. A new class of antiviral agents, capable of inhibiting prenylation, have been developed for use in humans[50]. Lonafarnib is an oral prenylation inhibitor. A proof-of-concept phase 2A double-blinded randomized placebo-controlled study showed a decline in serum HDV-RNA levels in patients treated with lonafarnib (100 mg or 200 mg twice daily) compared with placebo. The

decline in virus levels was proportional to serum drug concentration. Two-thirds of patients treated with the higher dose had a viral rebound and ALT flare after the end of treatment[51].

Triple combination therapy with lonafarnib, ritonavir, and peg-IFN for 6 mo showed antiviral efficacy in patients with chronic HDV infection, as demonstrated in a recent study (NCT03600714; n = 26)[52]. At the end of treatment (week 24), 77% of patients achieved an important goal, the decrease of HDV RNA (> 2 log).

Recent studies are investigating the capacity of NAPs to block HBsAg release from infected hepatocytes by some non-immunostimulatory mechanism. NAPs clear circulating HBsAg by hindering the release of HBV and HDV particles. The REP-2139 is the first NAP selected for human studies. Unpublished interim results from an ongoing proof of concept trial have confirmed the ability of REP 2139 to achieve clearance of HDV-RNA in 50% of treated patients up to 12 mo after the end of treatment. The adverse events (pyrexia, chills, conjunctival hyperemia, headache, and asthenia) could represent a serious limit.

Further studies are needed to assess the efficacy and tolerability of NAPs. Bulevirtide and lonafarnib are antiviral agents in an advanced phase of investigation with a proven anti-HDV activity.

HEPATITIS C

HCV infection is one of the most important causes of chronic liver disease in the world. The primary goal of HCV therapy is the infection cure, achieving an SVR consisting of undetectable HCV-RNA. SVR also means normalization of transaminase, reduction of liver necro-inflammation and fibrosis, improvement in liver function, and reduction of HCC risk[53].

The approval of direct-acting antivirals (DAAs) in 2014 revolutionized the treatment of HCV, allowing nearly all patients to be cured. DAAs are highly effective and well-tolerated and represent the gold standard for the treatment of patients with chronic infection, in all stages of liver disease[54]. Most of the progression in HCV treatment is due to the possibility to treat many populations, once excluded for the discouraging side effects of IFN-based therapies. At present, high SVR rates are achieved in cirrhotic patients, HIV/HCV co-infected patients with chronic renal failure, or transplant patients. The efficacy is not undermined by the short duration of DAA therapy.

Thanks to the IFN-free regimens developed, the World Health Organization (WHO)'s goal for HCV infection is a 90% reduction in the incidence of new infections and reduction of HCV-related deaths by approximately 65% in 2030[55,56].

There are many available EMEA- and FDA-approved DAAs for HCV treatment, divided into three major classes based on their HCV proteins targets: Protease NS3/4A inhibitors (glecaprevir, grazoprevir, paritaprevir, simeprevir, and voxilaprevir), NS5A serine protease inhibitors (PIs) (daclatasvir, elbasvir, ledipasvir, ombitasvir, pribrentasvir, and velpatasvir), NS5B RNA-dependent RNA nucleoside polymerase (sofosbuvir), and non-nucleoside polymerase (dasabuvir) inhibitors[57]. NS3/4A PIs inhibit HCV polyprotein processing; NS5A inhibitors inhibit viral replication and assembly; NS5B polymerase inhibitors block HCV-RNA replication.

The HCV drug combinations available in Europe are sofosbuvir and velpatasvir in a single tablet administered once daily; sofosbuvir, velpatasvir, and voxilaprevir available in a combination product, glecaprevir, and pibrentasvir or grazoprevir and elbasvir available in a two-drug fixed-dose combination. The non-pan-genotypic combination of grazoprevir and elbasvir can also be used in patients infected with HCV genotype 1b. Pan-genotypic HCV drugs, in particular sofosbuvir plus velpatasvir and glecaprevir plus pibrentasvir, can be used even if the virus genotype is not available.

Contraindications to current DAAs are few. The pharmacological interactions are worthy of attention: Cytochrome P450/P-gp-inducing agents are often not switchable. The use of grazoprevir, glecaprevir, or voxilaprevir is allowed only in a contest of compensated cirrhosis (Child-Pugh B or C) and in patients without previous episodes of decompensation (EASL guidelines)[54]. Patients with decompensated cirrhosis and patients with compensated cirrhosis with a medical history characterized by previous episodes of decompensation should be treated with sofosbuvir and velpatasvir and ribavirin for 3 mo. If there are contraindications to the use of ribavirin or poor tolerance, a fixed-dose combination of sofosbuvir and velpatasvir for 24 wk without ribavirin can be used.

Two innovative and promising strategies are required and expected to obtain a global HCV elimination: A deep enhancement of screening and linkage to care for so-called "hard-to-reach" populations. The strengthening of new strategies to improve access to care for vulnerable and marginalized populations with HCV infection is becoming urgent[58]. Moreover, it has been demonstrated that a drastic decrease in HCV incidence requires proper education on injection safety, appropriate screening blood transfusions, and delivery of sterile syringes to people who inject drugs[55].

Political will and financing issues, thus, are essential to achieve elimination or a significant reduction in HCV infection prevalence and incidence.

Apart from the implementation of measures to reduce the prevalence of HCV infection, a big innovation in the HCV field is linked to a paper, published in 2020, that suggests DAA therapy as the prevention of HCV transmission. A single-arm trial evaluated short-course prophylactic therapy in patients (n = 30) receiving solid organ transplants from donors with HCV infection[59].

Prophylaxis with glecaprevir-pibrentasvir combined with the HCV entry blocker ezetimibe (from 6-12 h to 7 d after transplantation) showed prevention of chronic HCV infection in all 30 transplant recipients. This innovative study could help to reduce ethical concerns regarding the transplantation of HCV-infected organs into recipients without HCV.

In conclusion, the treatment with new oral DAA combinations is efficient and safe even in special populations and in patients with advanced liver disease or severe comorbidities. Eradication of HCV by 2030 is not a utopian goal if all countries direct efforts towards the same objective: Preventing transmission measures, improving screening programs (especially in marginalized populations without contact with healthcare services or incarcerated individuals), and enforcing educational approach among people who inject drugs who represent the most vulnerable group for HCV transmission.

Problem of DAA resistance in the treatment of hepatitis C

A large part of the responsibility for DAA failure, which involves a minority of patients (4%-5%), is the inadequate adherence to therapy. In some cases, resistance-associated variants (RAVs) directly cause relapse or viral breakthrough. RAVs affect all DAA classes but mostly occur in the NS5A region. RAVs arising after treatment are more difficult to treat; however, patients who fail to achieve SVR after first-line DAA therapy, respond to sofosbuvir/velpatasvir/voxilaprevir and glecaprevir/pibrentasvir [60].

HEPATITIS E

HEV infection has recently been known to represent a major health problem in industrialized countries, not only in developing countries. This peculiar virus, transmitted as a zoonotic infection (uncooked meat consumption) or through infected blood or blood products, is endemic in most high-income countries (mainly genotypes 3 and 4) and is an important cause of acute and chronic viral hepatitis, the latter affecting immunosuppressed patients (such as solid transplant recipients, patients with hematological malignancies receiving chemotherapy, and HIV infected patients). The incidence of acute HEV infection is about 3 million human infections *per* year worldwide. In the endemic areas, HEV is transmitted predominantly by the fecal-oral route while in the non-endemic areas, HEV is a foodborne illness. Immunocompetent individuals develop self-limiting acute icteric hepatitis. It is a mild form providing long-term protection. HEV is the leading cause of 1%-4% of acute viral hepatitis cases in the general population and 30% in pregnant women. Few patients have severe illnesses leading to fulminant hepatic failure. In immunocompromised patients, acute infections progress in over 60% of cases to chronicity and in 10% to liver cirrhosis. The presence of HEV-RNA for more than 12 wk stands for chronic HEV infection. HEV chronicity is most common among liver transplant recipients. Interestingly, chronic HEV infection has been reported to be associated with a variety of extra-hepatic manifestations, for example, neurological and renal (neuralgic amyotrophy, Guillain-Barré syndrome, and membranoproliferative and membranous glomerulonephritis) [61]. HEV can also act as a potential trigger of acute-on-chronic liver failure (ACLF) that causes a worsening of the liver function with clinical complications such as ascites and encephalopathy. Acute HEV infection is responsible for 3.2% of the cases of decompensation in patients with chronic hepatic disease, as shown in a large prospective study including 343 patients, with elevated mortality[62].

In chronic HEV infection, the reference tests for the confirmatory diagnosis are the detection of HEV-RNA in serum or stool samples by reverse transcriptase-polymerase chain reaction or loop-mediated isothermal amplification for a minimum of 3–6 mo duration[63]. Antibody tests are not useful in the diagnosis of chronic HEV.

There are no approved treatments for this infection although reduction of immunosuppression can lead to HEV clearance in one-third of patients. The study of Kamar *et al*[64] showed that reduction in immunosuppressive therapy that specifically targets T cells could promote HEV eradication in transplant recipients, in the face of a high risk of graft rejection[64]. However, dose reduction of immunosuppressive medications, particularly those targeting T lymphocytes, is the first-line therapeutic approach for solid organ transplant recipients with HEV; mycophenolate mofetil seems able to suppress viral replication.

The currently recommended drug for the treatment of severe acute and chronic hepatitis E in solid transplant recipients is off-label oral ribavirin. The EASL published guidelines (2018) recommended the use of ribavirin as a standard of care; however, the optimal ribavirin treatment regimen is not known. Ribavirin inhibits HEV-RNA replication by depleting guanosine triphosphate pools.

In a large, retrospective, multicenter case series authors showed that a 3 mo course of ribavirin is a proper strategy to obtain SVR in chronic HEV infection[65]. Ribavirin can cause anemia, in up to 50% of patients. Peg-IFN-α has been reported as a possible treatment in the setting of liver transplant recipients: Peg-IFN-alpha 2a administered to three liver transplant patients with chronic HEV infection for 2 mo, resulted in viral RNA clearance. Peg-IFN-γ with or without ribavirin has also been shown to be effective in the treatment of HEV-HIV patients and non-transplant immunosuppressed patients with hematological disorders[66]. IFN can be considered in liver transplant recipients and patients undergoing hemodialysis; IFN is contraindicated in many solid-organ transplant patients (kidney, heart, and lung) because of the risk of rejection.

Based on available data, a decrease in immunosuppression (if feasible) and ribavirin at a dose of 600-800 mg *per* day for 3 mo are recommended in patients with chronic HEV hepatitis. In the case of ACLF provoked by HEV, the efficacy of ribavirin is not clear. Ribavirin is contraindicated in pregnancy due to teratogenic potential, and can be suggested for pregnant women only in the last trimester of pregnancy.

Sofosbuvir was also reported to reduce HEV viral load without achieving SVR in seven chronic HEV-infected patients. Sofosbuvir has therapeutic activity against HEV with an additive effect to ribavirin. Sofosbuvir with ribavirin, in chronic HEV infection, promotes a reduction in HEV levels and ALT during therapy, but a rise has been documented after stopping treatment, confirming its inability to provide SVR in chronic HEV infections.

Further studies are needed to assess the efficacy and safety of Hecolin, the first vaccine produced by China, not approved yet for commercialization. This vaccine (30 mcg of purified recombinant hepatitis E antigen *per* dose) given at 0, 1, and 6 mo showed 100% efficacy in phase III clinical trial[67]. The safety and efficacy of the vaccine in patients with chronic liver disease and other populations, for example, immunosuppressed patients, are needed before recommended for its widespread use.

There is a clinical need for new therapeutic options for HEV in immunosuppressed patients. Large scale studies are required to understand the impact of some promising compounds such as 2'-C-methylguanosine, which suppresses the growth of HEV in cell cultures, zinc that could act as an adjuvant therapy in ribavirin resistant/relapsed HEV infections, and silvestrol that diminished fecal HEV-RNA in mice but has not been tested on humans[68,69].

CONCLUSION

Novel DAAs have shown an unimaginable efficacy of curing HCV to the point that the WHO defined it as an achievable target elimination of HCV by 2030. To pursue this goal, marginalized and stigmatized patients should be better engaged in the healthcare system to diagnose infection and encourage treatment adherence.

Concurrently with the approval of DAAs for HCV, starting in 2014, a renewed interest in pursuing curative therapies for HBV infection has been registered. Current NAs positively impact liver-related complications, ensuring a high level of safety and tolerability. The need for long, or even lifelong, therapy and the oncogenic role of HBV, fostered by integration into the hepatocytes genome, has increased the effort to develop definitive and curative therapy. Novel approaches to HBV cure are based on

new antivirals, targeting different steps of the HBV life cycle and immune modulators. Molecules acting on the HBV cycle have obtained encouraging results. Immune-based approaches are also emerging but must be further examined. Ideal and utopistic therapy should pursue a sterilizing aim, consisting of elimination of cccDNA or silencing its activity. A combination of traditional and new anti-HBV agents may favor HbsAg clearance and eliminate the cccDNA reservoir from the patient's liver, reducing the oncogenic effect.

About HDV, the refined knowledge of its life cycle has facilitated the study of some direct-acting agents, bulevirtide, lonafarnib, and NAPs, which are more effective than IFN-based therapies. In particular, bulevirtide is the first drug approved for the treatment of HDV in adults with compensated liver disease in Europe.

Severe side effects and limited effectiveness of current HEV therapies have also made it essential to identify new therapeutic approaches for HEV, especially in immunosuppressed individuals. Further studies are required to assess the efficacy of adjuvant zinc therapy, 2'-C-methylguanosine, sofosbuvir, or the natural compound silvestrol.

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ORIGINAL ARTICLE

Basic Study

Effect of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* supernatants on serotonin transporter expression in cells and tissues

Yi-Ming Chen, Ying Li, Xin Wang, Ze-Lan Wang, Jun-Jie Hou, Shuai Su, Wei-Long Zhong, Xin Xu, Jie Zhang, Bang-Mao Wang, Yu-Ming Wang

ORCID number: Yi-Ming Chen 0000-0001-9793-7876; Ying Li 0000-0002-7887-108X; Xin Wang 0000-0002-4727-1612; Ze-Lan Wang 0000-0002-2298-1968; Jun-Jie Hou 0000-0002-8129-5618; Shuai Su 0000-0002-2012-9704; Wei-Long Zhong 0000-0002-7139-169X; Xin Xu 0000-0001-8083-0095; Jie Zhang 0000-0002-8100-5369; Bang-Mao Wang 0000-0002-4702-9711; Yu-Ming Wang 0000-0002-0392-1150.

Author contributions: Wang YM, Wang BM and Zhang J conceived and designed the study; Chen YM and Wang ZL conducted the experiments; Li Y and Wang X rote the manuscript; Su S, Hou JJ, Zhong WL and Xu X analyzed data; Chen YM and Li Y contributed equally to this work; all authors read and approved the manuscript.

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Yi-Ming Chen, Ying Li, Xin Wang, Ze-Lan Wang, Jun-Jie Hou, Shuai Su, Wei-Long Zhong, Xin Xu, Jie Zhang, Bang-Mao Wang, Yu-Ming Wang, Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, Tianjin 300052, China

Corresponding author: Yu-Ming Wang, MD, Chief Doctor, Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, No. 154 Anshan Road, Heping District, Tianjin 300052, China. yumingwangbest@163.com

Abstract

BACKGROUND

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of irritable bowel syndrome (IBS). Whether the supernatants of these three probiotics can improve gastrointestinal sensation and movement by regulating the serotonin transporter (SERT) expression needs to be clarified.

AIM

To investigate whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants can upregulate SERT expression *in vitro* and *in vivo*.

METHODS

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A male Sprague-Dawley rat model of post-infectious irritable bowel syndrome (PI-IBS) was established and the rats were treated with phosphate-buffered saline (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

RESULTS

The levels of SERT at post-treatment 12 and 24 h were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, SERT expression in groups B, C, and D was significantly higher than

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that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was only found in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in SERT expression between the groups in the last week ($P > 0.05$).

CONCLUSION

The supernatants of B. *subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS.

Key Words: *Bacillus subtilis* supernatant; *Enterococcus faecalis* supernatant; *Enterococcus faecium* supernatant; Serotonin transporter expression; Irritable bowel syndrome

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Core Tip: Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms and changes in bowel habits. Serotonin and serotonin transporter (SERT) play significant roles in the development of IBS. *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS. Here, we explored the effects of *B. subtilis*, *E. faecium,* and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo,* highlighting the significance of the regulation of SERT expression in the treatment of IBS patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms, such as abdominal pain and changes in bowel habits[1]. According to the Rome IV criteria, IBS is divided into four subtypes: IBS with predominant constipation (IBSC), IBS with predominant diarrhea (IBSD), IBS with mixed bowel habits (IBSM), and unclassified IBS (IBSU)[2]. With a prevalence varying between 5% and 10% worldwide, IBS can reduce the health-related quality of life and lower work productivity. Some symptoms of IBS such as an abnormal psychological state, anxiety, and depression may impose an economic burden on individuals, healthcare systems, and society[3,4]. The etiology of IBS is complex and still elusive. The widely recognized pathogenic mechanisms include gastrointestinal dysmotility, varied visceral sensitivity, brain-gut axis disorder, gut microenvironment, and psychosocial/psychosomatic behaviors[3]. For decades, the significant decrease in expression of the serotonin transporter (SERT) in the intestinal mucosa has been considered one of the most important pathophysiology in the development of IBS, leading to the gastrointestinal motility disorders[5-7]. SERT terminates serotonin or 5-hydroxytryptamine (5-HT) activity by combining with it from the interstitial space. Reduced SERT expression and function may result in excess 5-HT, along with motor, sensory, and secretary dysfunctions of the gut, which in turn leads to the development of diarrhea and abdominal pain associated with the pathogenesis of IBS[8,9]. In addition, alterations in 5-HT metabolism have been postulated to play a role in the pathogenesis of IBS[10,11]. Overall, 5-HT and SERT contribute significantly to the development of IBS[12].

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS[13-16]. These probiotics can alleviate IBS symptoms such as decreasing the intensity of abdominal pain and discomfort and reducing stool frequency. Our previous study revealed that *Lactobacillus rhamnosus GG* supernatants can upregulate the expression

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level of SERT in epithelial cells and colon tissues in rats with post-infectious IBS (PI-IBS)[7,17]. We also demonstrated that *Lactobacillus acidophilus* and *Bifidobacterium longum* supernatants have similar effects on SERT expression in intestinal epithelial cells[18]. However, whether the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression still needs to be clarified.

The current research explored the effects of *B. subtilis*, *E. faecium,* and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial culture

B. subtilis (CGMCC 1.3358), *E. faecium* (CGMCC 1.2136), and *E. faecalis* (CGMCC 1.2135) were obtained from China General Microbiological Culture Collection Center (Beijing, China). These bacteria were respectively inoculated into the corresponding liquid medium and cultured at 37 °C for 24 h (*B. subtilis*: nutrient broth medium, HB0105, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecium*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecalis*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China). Then they were diluted in the corresponding media, and continued to be cultured to reach the logarithmic stage with an optical density (OD) of 0.5 detected at a wavelength of 600 nm. The supernatants were collected by centrifugation (5000 g, 10 min) and filtered twice through a 0.22 μm filter.

Campylobacter jejuni 81-176 (BAA-2151; ATCC, Manassas, VA, United States) was grown on Skirrow's selective medium (Columbia Agar Base, Oxoid CM0331) at 42 °C for 24 h. Bacterial colonies were obtained with an inoculating loop and diluted with culture medium until reaching a concentration of 10^{10} CFU/mL. Determination of bacterial concentration was carried out by the conventional plate-counting method.

Cell culture

Caco-2, a human epithelial colorectal adenocarcinoma cell line, was grown in Eagle's minimal essential medium (MEM; Gibco, New York, NY, United States) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% nonessential amino acids at 37 °C. The human colonic epithelial carcinoma cell line, HT-29, was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY, United States) supplemented with 10% FBS and 1% nonessential amino acids at 37 °C. The cells were incubated with the culture supernatants of *B. subtilis, E. faecium*, and *E. faecalis* diluted in MEM/DMEM (dilution ratios: 1:100, 1:50, and 1:20, respectively) for 12 and 24 h and classified as the 1:100 group, 1:50 group, and 1:20 group, respectively.

Animal studies

Sixty-six male Sprague-Dawley rats (270-310 g) were maintained in a room at 22 ± 1 °C under a 12-h light:12-h dark cycle in the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Tianjin, China). Rats in all groups were given the same housing conditions and diet. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. The animal protocol was designed to minimize pain or discomfort to the animals. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (IACUC Protocol No. IRM-DWLL-2021142).

Intervention and evaluation of a rat model of PI-IBS

Rats were randomly divided into a PI-IBS group (n = 52) and control group (n = 14). Rats in the PI-IBS group were given 10^{10} CFU/mL *C. jejuni* for 7 days, while those in the control group were given phosphate-buffered saline (PBS) for 7 days. The rats in both groups were fed separately. *C. jejuni* stool culture and biochemical tests were carried out to evaluate the infection phase of rats in the PI-IBS group. Four rats were randomly chosen from the PI-IBS and control group to perform the intestinal motility test with carbon solution gavage. The specific experimental process was previously described by Wang *et al*[19]. After evaluation of the rat model of PI-IBS, the remaining rats were randomly assigned to four different experimental groups (n = 12 for each group): Group A, PBS (i.g); Group B, *B. subtilis* supernatant (i.g); Group C, *E. faecalis* supernatant (i.g); Group D, mixed supernatant *of B. subtilis* and *E. faecalis* with a dilution of 1:1 (i.g). These rats were treated for 4 wk, and changes in general and fecal

states were observed and recorded. Besides, three rats from each group were sacrificed, and protein expression levels of SERT in colonic tissues were detected weekly. The rats were fed 10% activated carbon suspension after fasting for 24 h and were killed 1 h later. The intestinal section from pyloric to terminal rectum was removed immediately. The total length of intestinal tract and the propelling distance of activated carbon were measured. Intestinal transit rate = propelling distance of activated carbon/total length of intestinal tract.

quantitative PCR

Total RNA was extracted from Caco-2 and HT-29 cells with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, United States). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The raw materials for PCR synthesis included cDNA, 2x iQSYBR Green Supermix (Thermo Fisher Scientific), primers, and double-distilled water. The primers used for quantitative PCR (qPCR) are listed in Table 1. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted from HT-29 cells, Caco-2 cells, and colonic tissues of rats using radio-immunoprecipitation assay lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Solarbio Science & Technology Co., Ltd., Beijing, China). A total of 40 g protein samples were isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isolated protein was transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk powder at room temperature for 1 h, and incubated with primary rabbit polyclonal antibodies (SERT, Cat. No. ab102048, Abcam, Cambridge, UK; β-actin, Cat No. KM9001, Tianjin Sungene Biotech Co., Ltd., Tianjin, China) at 4 °C overnight. The PVDF membranes were incubated with diluted secondary antibody (LK2001, Tianjin Sungene Biotech Co.) at room temperature for 1 h. Then the membranes were washed with Tris-buffered saline with 0.1% Tween® 20 Detergent and detected using an enhanced chemiluminescence kit (BB-3501; Amersham, Buckinghamshire, UK). The Bio-Rad Image Analysis System (Bio-Rad Laboratories) was utilized to visualize the protein bands. The protein quantitative analysis was undertaken using ImageJ software. The relative expression was calculated using the ratio of the gray value of protein band to that of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

The Student's *t*-test was used to assess differences between two groups. All statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of B. subtilis supernatant on the mRNA and protein expression levels of SERT in Caco-2 and HT-29 cells

Caco-2 and HT-29 cells were treated with supernatants of *B. subtilis* from 1:100, 1:50, and 1:20 groups. SERT mRNA and protein expression levels in Caco-2 and HT-29 cells were detected by qPCR and western blotting, respectively. SERT mRNA levels at both 12 and 24 h after treatment were significantly increased in the 1:50 and 1:20 groups in Caco-2 cells (Figure 1 Caco-2 A&B, all $^{a}P < 0.05$) compared with those in the control group. Increased SERT protein expression was found in the 1:20 group at 12 h after treatment (Figure 1 Caco-2C, $^{a}P < 0.05$). Both SERT mRNA and protein levels increased at 24 h (Figure 1 Caco-2B&D, $^{a}P < 0.05$). The mRNA and protein expression levels of SERT in the 1:20 group were significantly higher than those in the 1:100 group at 12 and 24 h ($^{a}P < 0.05$), which indicated that the effect of *B. Subtilis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT protein expression in HT-29 cells was markedly elevated in the 1:50 and 1:20 groups at 12 h compared with that in the control group, and the level of SERT proteins in the 1:20 group was higher than that of the 1:100 and 1:50 group (Figure 1 HT-29C, $^{a}P < 0.05$). However, there was no significant difference in SERT mRNA level at 12 h among the treatment and control groups (Figure 1 HT-29A, $P > 0.05$). By contrast,

Table 1 Primer sequences for RT-PCR

Gene	Sequence (5'-3')
rGAPDH	Forward: 5'-CCATCAACGACCCCTTCATT-3'; Reverse: 5'-GACCAGCTTCCCATTCTCAG-3'
rSERT	Forward: 5'-ACTGTTACCAAGATGCCCTG-3'; Reverse: 5'-ATCTTCATTCCTCATCTCCGC-3'
hGAPDH	Forward: 5'-ACA GCA ACT CCC ATT CTT-3'; Reverse: 5'-TCC AGG GTT TCT TAC TCC-3'
hSERT	Reverse: 5'-AAT GGG TAC TCA GCA GTT CC-3'; Reverse: 5'-CCA CAG CAT AGC CAA TCA C-3'

hGAPDH: Human glyceraldehyde-3-phosphate dehydrogenase; hSERT: Human serotonin transporter; rGAPDH: Rat glyceraldehyde-3-phosphate dehydrogenase; rSERT: Rat serotonin transporter.

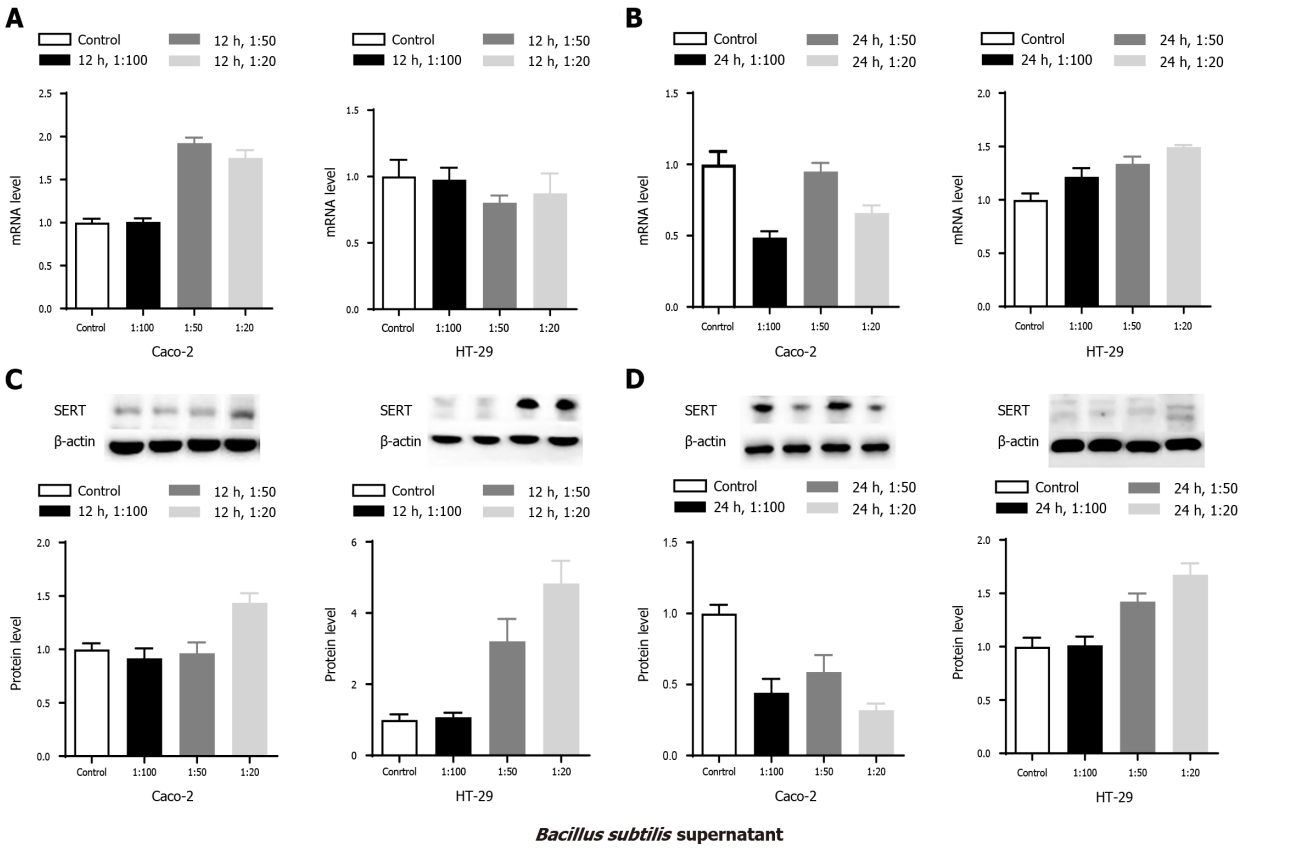


Figure 1 Effects of *Bacillus subtilis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

significant reductions in the mRNA and protein levels of SERT were observed among the treatment groups at 24 h (Figure 1 HT-29-B&D, all $^{a}P < 0.05$).

Effects of E. faecium supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecium* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were performed. The experimental results showed that there were no significant differences in the mRNA and protein levels of SERT at 12 h among the mentioned groups (Figure 2 Caco-2-A&-C, $^{a}P > 0.05$). SERT mRNA and protein levels were significantly upregulated in the 1:50 and 1:20 groups compared with the control group at 24 h (Figure 2 Caco-2-B&-D, $^{a}P < 0.05$), indicating that the effects of *E. faecium* supernatant on SERT mRNA and protein expression were time-dependent. Besides, SERT protein level in the 1:20 group was higher than that in the 1:50 and 1:100 group at 24 h (Figure 2 Caco-2D, $^{a}P < 0.05$), demonstrating that the effect of *E. faecium* supernatant was concentration-dependent.

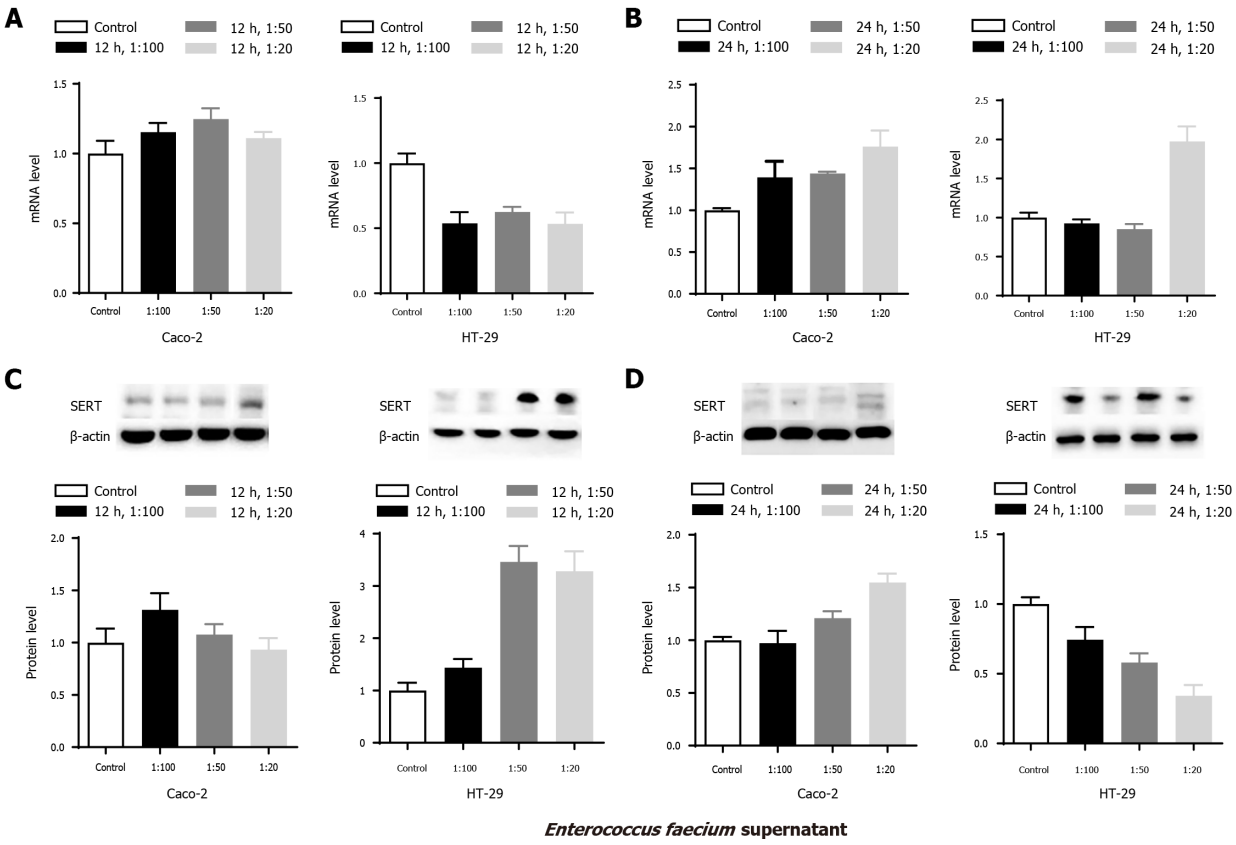


Figure 2 Effects of *Enterococcus faecium* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

SERT protein levels in the 1:50 and 1:20 groups were markedly higher than those in the control group in HT-29 cells at 12 h (Figure 2 HT-29C, $^{a}P < 0.05$). However, SERT mRNA levels in the three treatment groups were dramatically reduced at 12 h (Figure 2 HT-29-A, $^{a}P < 0.05$). Furthermore, compared to the control group, SERT mRNA level was notably elevated in the 1:20 group at 24 h (Figure 2 HT-29B, $^{a}P < 0.05$), while SERT protein level was significantly reduced in the 1:50 and 1:20 groups at 24 h (Figure 2 HT-29-D, $^{a}P < 0.05$).

Effects of mixed supernatant of B. subtilis and E. faecium on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To further investigate the abovementioned results, Caco-2 and HT-29 cells were stimulated with the mixed supernatants of *B. subtilis* and *E. faecium* in the 1:1 group. Compared with the control group, SERT mRNA level was upregulated in the 1:20 group in Caco-2 cells at 12 h (Figure 3 Caco-2 A, $^{a}P < 0.05$), and SERT protein levels were significantly elevated in the three groups at 12 h (Figure 3 Caco-2 C, $^{a}P < 0.05$). However, there were no significant differences in the mRNA and protein levels of SERT among these groups at 24 h (Figure 3 Caco-2 B&D, $P > 0.05$).

The expression of SERT mRNA in HT-29 cells was upregulated in the 1:100 and 1:20 groups at 12 h (Figure 3 HT-29A, $^{a}P < 0.05$) compared to the control group. An increased level of SERT protein was found in the 1:20 group at 12 h, whereas a significantly decreased level was observed in the 1:50 group compared with the control group (Figure 3 HT-29C, $^{a}P < 0.05$). Furthermore, compared with the control group, SERT mRNA levels were attenuated in the 1:100 and 1:20 groups at 24 h, whereas SERT protein levels were decreased in all the three treatment groups (Figure 3 HT-29 B&D, $^{a}P < 0.05$).

Effects of E. faecalis supernatant on mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecalis* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were conducted. SERT mRNA

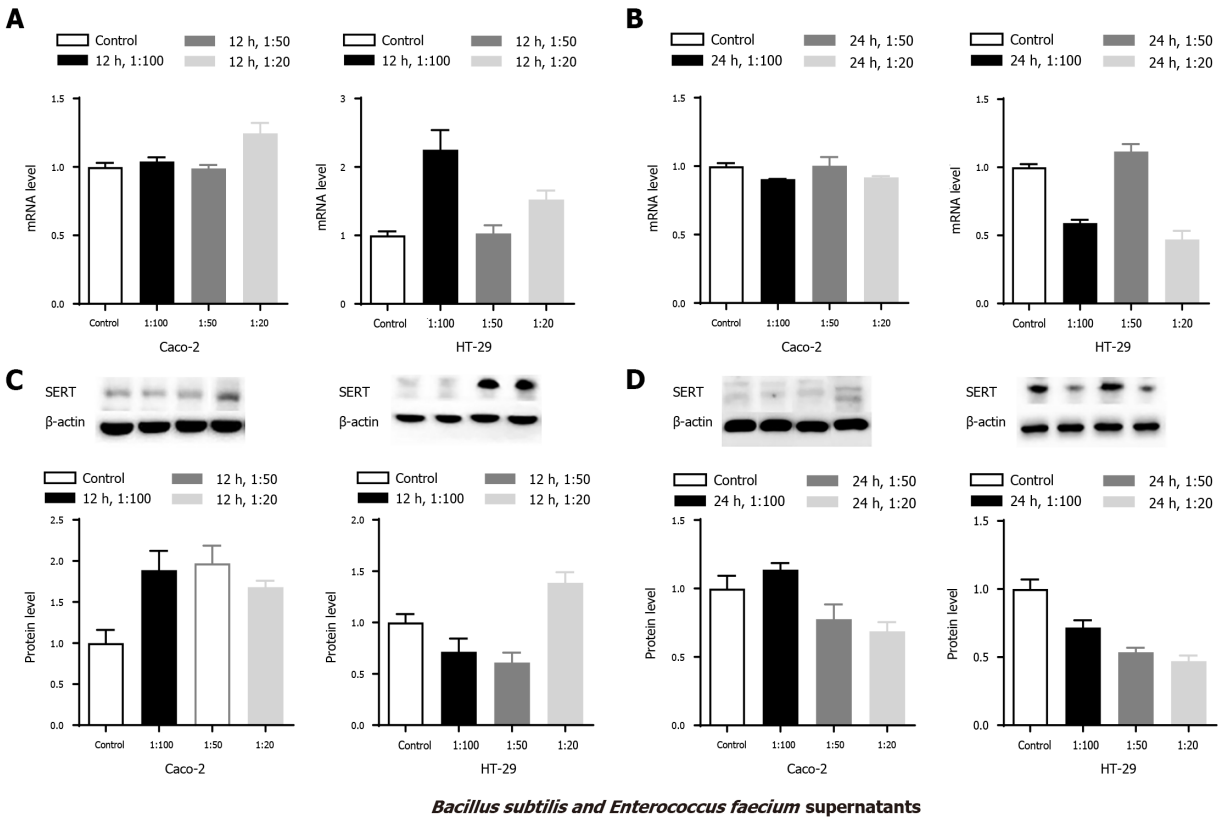


Figure 3 Effects of *Bacillus subtilis* and *Enterococcus faecium* supernatants on serotonin transporter mRNA and protein expression in Caco2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

levels in Caco-2 cells in the three treatment groups were significantly increased at 12 h (Figure 4 Caco-2 A, $^{a}P < 0.05$). Western blot analysis showed that there were no significant differences in SERT protein levels among the three treatment groups at 24 h, whereas a decreased level was observed in the 1:20 group at 12 h compared to the other two groups (Figure 4 Caco-2 C, $^{a}P < 0.05$). In addition, increased SERT mRNA level was only found in the 1:50 group at 24 h (Figure 4 Caco-2-B, $^{a}P < 0.05$). Similarly, the expression level of SERT protein was markedly upregulated in the 1:50 and 1:20 groups at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$). SERT protein level in 1:20 group was significantly higher than that in the 1:50 and 1:100 groups at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$). Besides, SERT protein level in the 1:50 group was also higher than that in the 1:100 group at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$), indicating that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT mRNA level was in steady-state in HT-29 cells at 12 h among the treatment groups (Figure 4 HT-29A, $P > 0.05$), while western blot analysis showed that the SERT mRNA level was significantly elevated in all three groups (Figure 4 HT-29-C, $^{a}P < 0.05$) compared to the control group. Additionally, the expression level of SERT protein in the 1:50 group was markedly higher than that in the 1:20 group at 12 h (Figure 4 HT-29C, $^{a}P < 0.05$). SERT mRNA level was markedly increased in the 1:20 group at 24 h compared to the 1:100 group (Figure 4 HT-29B, $^{a}P < 0.05$). Similarly, the levels of SERT protein were significantly elevated in the 1:20 and 1:50 groups at 24 h. Meanwhile, the increase in SERT mRNA expression in the 1:20 and 1:50 groups was noticeably higher than that in the 1:100 group at 24 h (Figure 4 HT-29-D, $^{a}P < 0.05$), confirming that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent in HT-29 cells.

The construction and evaluation of a rat model of PI-IBS

To further verify the effect of culture supernatant of probiotics in treating IBS, we constructed a rat model of PI-IBS. Before intervention, all rats were negative for *C. jejuni* stool culture and biochemical tests. Fifty-two of the rats were used to establish a rat model of PI-IBS as the PI-IBS group. The results of fecal bacteriological culture and

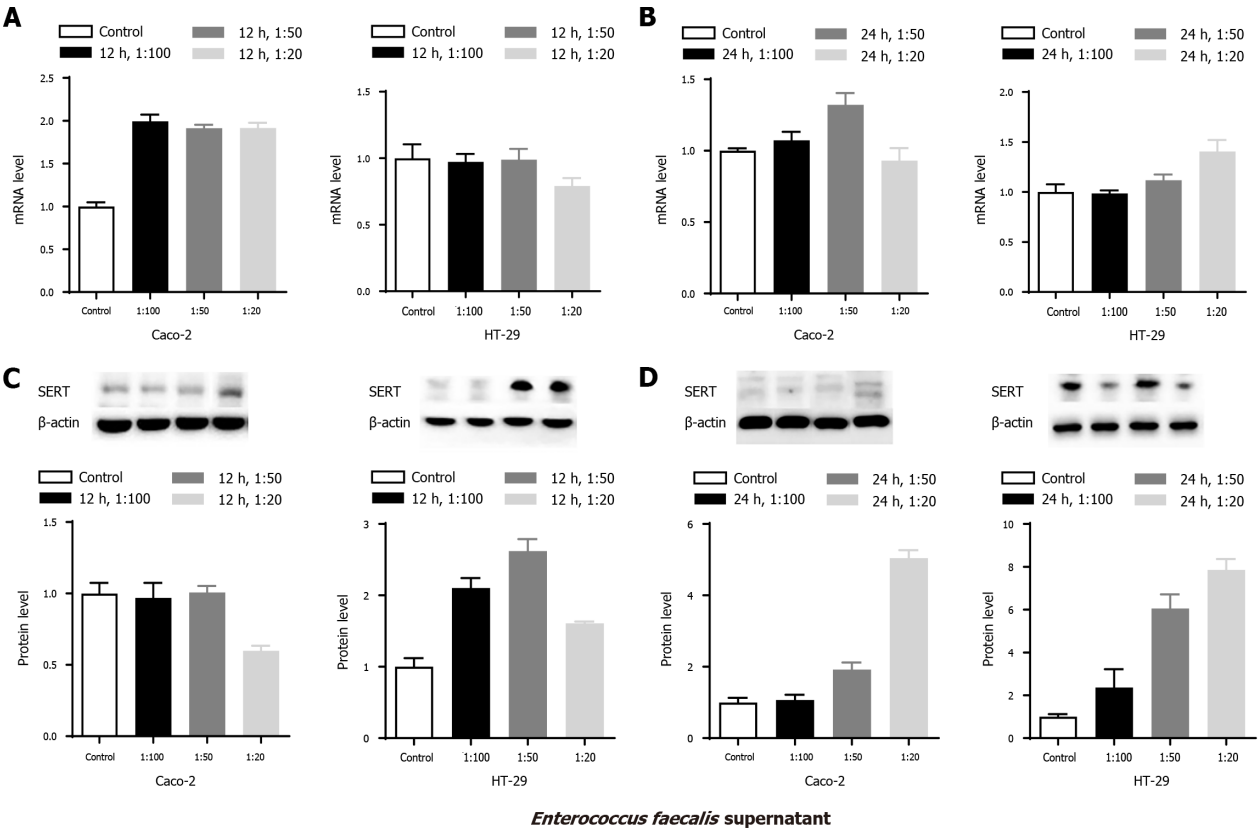


Figure 4 Effects of *Enterococcus faecalis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells.
A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

biochemical tests were positive on the 7th d after gavage in the PI-IBS group. Twenty-four rats in the control group had lively spirits, quick reactions, bright hair, normal diet, and normal urine and feces. The activities of rats in the PI-IBS group were reduced, lack of luster was observed in the hair of rats, and their appearance was thinner compared with the control group (Figure 5A). However, there were no significant differences between the PI-IBS and control group from 5th wk to 7th wk after gavage. The negative rates of *C. jejuni* fecal culture and biochemical tests (Figure 5B) in the PI-IBS group were higher than 95% at the 7th wk and 9th wk. After 8 wk of *C. jejuni* gavage, four rats from each group were randomly selected for model's evaluation, and the rat model of PI-IBS was confirmed to be successfully constructed. The intestinal transport speed in the PI-IBS group were higher than those in the control group and the expression level of SERT protein in the PI-IBS group were lower than those in the control group (Figure 5C and 5D, $^{a}P < 0.05$).

The efficacy of probiotic therapy on the rat model of PI-IBS

After administration of several probiotic supernatants (group B: *B. subtilis* supernatant; group C: *E. faecalis* supernatant; group D: mixed supernatants of *B. subtilis* and *E. faecalis* with dilution ratio of 1:1), the expression level of SERT protein was further detected to evaluate the intestinal motility of rats.

SERT protein expression in groups C and D were elevated compared with that in the model group (group A) during the 1st wk, and the efficacy of group C was superior than that of group B (Figure 6 w_1, $^{a}P < 0.05$), demonstrating that the efficacy of *E. faecalis* supernatant was superior than *B. Subtilis* supernatant in the 1st wk. The expression levels of SERT protein in groups B, C, and D were markedly higher than that in group A in the 2nd wk (Figure 6 w_2, $^{a}P < 0.05$), and the increase was only observed in the group D in the 3rd wk (Figure 6 w_3, $^{a}P < 0.05$). Besides, the expression level of SERT protein in group D was greater than that in groups B and C in the 2nd wk and 3rd wk (Figure 6 w_2, w_3, $^{a}P < 0.05$), reflecting that the efficacy of mixed supernatant was more significant than single supernatant in the 2nd wk and 3rd wk. Meanwhile, the ratio of expression level of SERT protein in group B to that of group A was elevated from the 1st wk to the 2nd wk, indicating that the efficacy of *B. Subtilis*

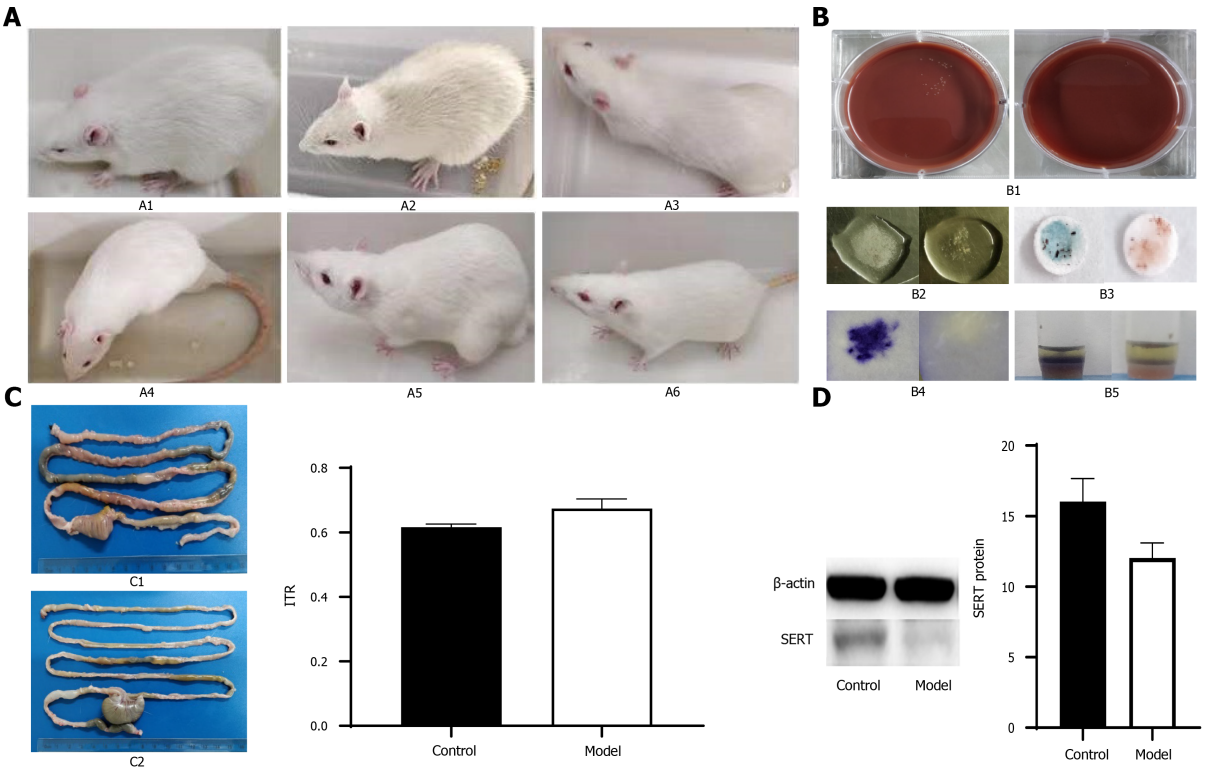


Figure 5 Rat model of post-infectious irritable bowel syndrome. A: Appearance changes of experimental rats; A1: model rats on the 7th day after gavage; A2: the 14th day after gavage; A3: The 28th d after gavage; A4: the 42nd d after gavage; A5: the 56th day after gavage; A6: the normal control group; B: Fecal culture and biochemical detection of *Campylobacter jejuni* B1: *Campylobacter jejuni* culture; B2: catalase test; B3: indole acetate test; B4: oxidase test; e: sodium hippurate hydrolysis test (a-e pictures show that the left picture is positive, the right picture is negative); C: Intestinal transport (ITR) experiment C1: Pylorus to rectal segment in model group; C2: Pyloric to rectal segment in control group; C3: Comparison of ITR histogram ($^aP < 0.05$); D: Expression of serotonin transporter (SERT) protein in the colon during the evaluation period after model evaluation and measurement of ITR experiment. Colon tissue was taken for western blotting to measure SERT level, and quantitative analysis of protein band gray level was used ($^aP < 0.05$).

supernatant was time-dependent. Besides, the ratio of expression level of SERT protein in group C to that of group A was reduced from the 2nd wk to the 3rd wk, representing that the efficacy of *E. faecalis* supernatant was not significant for long-term. There was no significant increase in the expression levels of SERT protein among the treatment groups at the end of the last week (Figure 6 w_4), demonstrating that the long-term efficacy of probiotic therapy was insignificant.

DISCUSSION

IBS is characterized by recurrent abdominal pain, and is associated with a change in stool frequency[3]. At present, there is no effective medical treatment for relieving the symptoms of IBS. Probiotic therapy is extensively applied in supporting the treatment of a broad range of gastrointestinal diseases, and has shown a modest effect on ameliorating the symptoms of IBS[20-23]. *L. rhamnosus GG*, *L. acidophilus*, *Bifidobacterium animalis subspecies lactis*, and *B. longum* are commonly used probiotics that can alleviate the symptoms of IBS[24-36]. A review of four clinical studies, which enrolled 214 IBS patients to evaluate the efficacy of a combination of *B. subtilis* and *E. faecium*, concluded that the combined therapy had a more significant effect on relieving abdominal pain without adverse events[16].

Previous animal studies have demonstrated that the combination of *E. faecium* and *B. subtilis* enhanced intestinal barrier function, increased the expression levels of zonula occludens-1 protein and occludin, and improved gut microbiota with a higher amount of *Lactobacilli*[37]. The expression level of ileal mucin 2 gene and bacteria population were increased in chickens intervened with *B. subtilis* in the 1st wk[38]. Besides, heat-killed *E. faecalis* EF-2001 has shown protective effects on a mouse model of colitis[39]. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

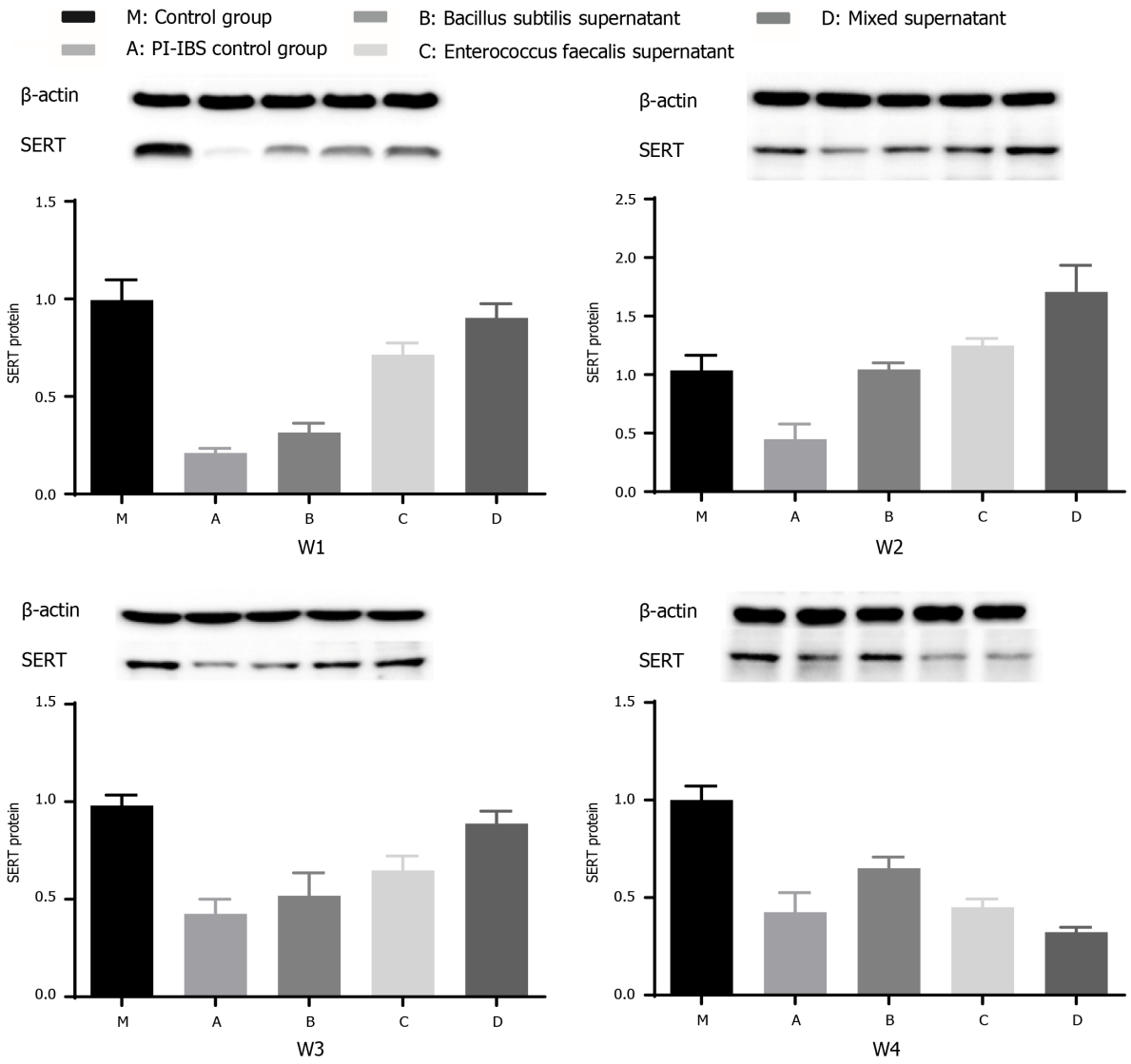


Figure 6 Effects of different supernatants of probiotics on serotonin transporter protein expression in rat intestinal tissues. Quantitative analysis of serotonin transporter (SERT) protein levels in the 1st wk (W1), 2nd wk (W2), 3rd wk (W3), and 4th wk (W4) analyzed by western blotting.

Previous studies have demonstrated that the supernatants of *L. rhamnosus GG*, *L. acidophilus*, and *B. longum* can upregulate the expression of SERT protein in epithelial cells and PI-IBS rats[7,17,18]. SERT, a regulator of the level of 5-HT, plays a significant role in the pathogenesis of IBS and can serve as a novel therapeutic target for IBS. In the present study, we found that the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* upregulated the mRNA and protein levels of SERT in intestinal epithelial cells, and the trend of mRNA and protein expression changes were similar. The expression level of SERT protein was upregulated in the intestinal tissues in a rat model of PI-IBS receiving probiotic therapy. Meanwhile, the efficacy of combined supernatants of *B. subtilis* and *E. faecalis* was superior to a single supernatant.

The mRNA and protein levels of SERT at 12 and 24 h were significantly increased in Caco-2 cells treated with supernatant of *B. subtilis*. *E. faecium* supernatant upregulated the SERT mRNA level in Caco2 cells at 24 h, and the trend of SERT protein level alteration was similar to that of SERT mRNA level. The results were similar in HT-29 cells stimulated with *B. subtilis* supernatant or *E. faecium* supernatant. SERT protein level was elevated at 12 h and reduced at 24 h. Thus, we speculated that upregulation of SERT expression in HT-29 cells intervened with *B. subtilis* or *E. faecium* supernatant was significant in the short-term. Moreover, we explored the efficacy of combination of *B. subtilis* and *E. faecium* supernatants on SERT expression. We found that the mRNA and protein levels of SERT were elevated at 12 h in Caco-2 and HT-29 cells treated with mixed supernatants of *B. Subtilis* and *E. faecium*, and the trend of SERT protein expression was similar to that of mRNA expression. Furthermore, our results confirmed the efficacy of mixed supernatants in relieving the symptoms of IBS, which

was consistent with the findings of a previous study[14].

Besides, increased mRNA and protein levels of SERT were observed in Caco-2 and HT-29 cells treated with *E. faecalis* supernatant for 12 and 24 h. The upregulation of SERT expression treated with *E. faecalis* supernatant could decrease intestinal motility, confirming that ProSymbioflor containing *E. faecalis* and *E. coli* was effective on relieving the symptoms of IBS patients[15].

We also assessed the effects of mixed supernatants of *B. subtilis* and *E. faecalis* with dilution of 1:1 on the expression level of SERT in intestinal tissues in the rat model of PI-IBS. Western blot analysis revealed that SERT protein expression was upregulated in the three therapeutic groups except for the last week. The efficacy of mixed supernatants was superior than that of single supernatant in the first 3 wk. The abovementioned results indicated that the *in vivo* regulatory mechanisms might be more complicated. Due to the acidic environment in the stomach, the function of active ingredients in the supernatants might be attenuated, which might justify the weak efficacy of the supernatants in the last week. Thus, establishing a rat model of PI-IBS motivated us to explore a new approach for protecting important organs from possible damages.

The change in trend of mRNA and protein expression was similar, and the difference in SERT expression level between Caco-2 cells and HT-29 cells might be attributed to different types of cells. Occasionally, SERT protein level alterations in the stimulated groups were not identical to that of the mRNA level in the following conditions. First, the difference between reductions or no changes in the SERT mRNA level and significantly increased SERT protein expression could be explained by the fact that the process of mRNA transcription occurred earlier than protein translation, mRNA was easier degraded than protein, and the status of mRNA was less stable than protein. Second, the inconsistent results regarding an increased SERT mRNA level and a reduced SERT protein could be interpreted by unknown factors of supernatants that might be involved in the process of translation from mRNA to protein. Third, the non-change trend of SERT protein expression was in contrast with the increased SERT mRNA level, which was illustrated by the fact that the expression process of SERT protein was slower and more complicated.

The efficacy of the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* was found time-dependent, which might help guide physicians to provide continuous treatment for patients with IBS in clinical practice. Moreover, the SERT expression level, which was regulated by the supernatants in a concentration-dependent manner, could be assessed by possible substances, such as bacterial components or small-molecule proteins regulating the SERT expression level. Such a soluble protein, p40, derived from LGG supernatant, could transactivate epidermal growth factor receptor (EGFR) signaling to reduce cytokine-induced apoptosis and epithelial barrier dysfunction *in vivo* and *in vitro*[40]. Benmansour *et al*[41] showed that EGF could upregulate the expression level of SERT in intestinal epithelial cells by activating the activator protien-1 (AP-1), and further interfering with the uptake of 5-HT. Cui *et al*[42] found that EGF could upregulate the expression level of SERT *via* EGFR signaling pathway. Other factors influencing the expression level of SERT include SERT gene polymorphisms, immune cells, inflammatory cytokines, microRNA 16 (MiR-16), and MiR-545[12]. *B. subtilis* has a highly adaptable metabolism and efficient protein expression and secretion system[43], including alkaline phosphatase and thermostable β-galactosidase[43]. A number of studies have demonstrated that *B. subtilis* and its conditioned media induced cytoprotective heat shock proteins in Caco-2 cells to protect colonic epithelial cells from damages[44-46]. Thus, it deserves to explore which specific factors in the supernatants of *B. subtilis*, *E. faecalis,* and *E. faecium* could regulate the expression level of SERT. The expression level of SERT was upregulated after stimulation with *B. subtilis* or *E. faecalis* supernatants for 12 and 24 h. However, the increase on the expression level of SERT was only observed in the cells stimulated with *E. faecium* supernatant at 24 h, and further research indicated that different potential active ingredients from probiotic supernatants might be effective at different time points or *via* different signaling pathways.

CONCLUSION

In conclusion, we found that the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* could upregulate the expression level of SERT in intestinal cells, and the effect of combined supernatants of *B. subtilis* and *E. faecalis* was superior than that of single supernatant on the expression level of SERT in colonic tissues in the rat model of PI-

IBS. Additionally, 5-HT and SERT showed to play important roles in the development of IBS, thus, these proteins were significant to treat IBS patients with lower expression level of SERT or with symptoms of diarrhea. In the next research, we will attempt to explore which effective ingredients in the probiotic supernatants can regulate the SERT expression, and will also find out signaling pathways modulating the SERT expression.

ARTICLE HIGHLIGHTS

Research background

It is worthwhile to explore which effective ingredients in the supernatants of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* and which signaling pathways are regulating serotonin transporter (SERT) expression further.

Research motivation

The supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS. And combined supernatants of *B. subtilis* and *E. faecalis* was more efficacious than single supernatant.

Research objectives

The levels of SERT (at post-treatment 12 and 24 h) were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, the SERT expression in groups B, C and D was significantly higher than that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was found only in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in the SERT expression between the groups in the last week ($P > 0.05$).

Research methods

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A rat (male Sprague-Dawley rat) model of post-infectious irritable bowel syndrome (PI-IBS) was constructed and the rats were treated with PBS (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

Research results

The present study aimed to explore whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants could upregulate SERT expression in *vitro and in vivo.*

Research conclusions

5-HT and SERT contribute significantly to the development of IBS. Whether the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression needs to be clarified. The research is significant to the treatment of IBS patients with lower expression level of SERT or with symptoms of diarrhea.

Research perspectives

IBS is a functional gastrointestinal disorder, of which the onset and development are associated with serotonin and SERT. Recent studies have shown that *B. subtilis*, *E. faecium*, and *E. faecalis* play important roles in the clinical treatment of IBS. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

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ORIGINAL ARTICLE

Basic Study

Connective tissue growth factor expression hints at aggressive nature of colorectal cancer

Ishrat Parveiz Bhat, Tahseen Bilal Rather, Irfan Maqbool, Gowhar Rashid, Kulsum Akhtar, Gulzar A Bhat, Fazl Q Parray, Besina Syed, Ishrat Younas Khan, Mohsin Kazi, Muhammad D Hussain, Mudassar Syed

ORCID number: Ishrat Parveiz Bhat 0000-0003-0663-6926; Tahseen Bilal Rather 0000-0002-1363-6601; Irfan Maqbool 0000-0003-1015-9536; Gowhar Rashid 0000-0002-6321-3877; Kulsum Akhtar 0000-0003-0546-4835; Gulzar A Bhat 0000-0003-0653-9931; Fazl Q Parray 0000-0003-3657-7808; Besina Syed 0000-0001-9886-1113; Ishrat Younas Khan 0000-0002-5179-5910; Mohsin Kazi 0000-0002-5611-0378; Muhammad D Hussain 0000-0003-1063-8203; Mudassar Syed 0000-0001-9288-5077.

Author contributions: Bhat IP completed the main experimental work, drafted the manuscript and analyzed the data; Rather TB helped in block collection for IHC experiments, revised the manuscript; Maqbool I contributed to experimental work; Rashid G contributed to patient sample collection; Akhtar K contributed to data collection and methodology design; Bhat GA gave directions in data analysis and helped in proofreading; Parray FQ provided access for patient sample and data collection; Syed B was involved in Immunohistochemical analysis of tissue samples; Khan IY contributed in IHC experimental work; Kazi M contributed to manuscript revision and scientific editing; Hussain MD contributed to manuscript language editing

Ishrat Parveiz Bhat, Tahseen Bilal Rather, Irfan Maqbool, Gowhar Rashid, Kulsum Akhtar, Gulzar A Bhat, Mudassar Syed, Department of Clinical Biochemistry, Sher-I-Kashmir Institute of Medical Sciences, Srinagar 190011, Jammu and Kashmir, India

Fazl Q Parray, Department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences, Srinagar 190011, Jammu and Kashmir, India

Besina Syed, Ishrat Younas Khan, Department of Pathology, Sher-I-Kashmir Institute of Medical Sciences, Srinagar 190011, Jammu and Kashmir, India

Mohsin Kazi, Department of Pharmaceutics, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia

Muhammad D Hussain, Department of Pharmaceutical and Biomedical Sciences, California Health Sciences University, California, CA 93612, United States

Corresponding author: Mudassar Syed, PhD, Full Professor, Department of Clinical Biochemistry, Sher-I-Kashmir Institute of Medical Sciences, Soura Srinagar Kashmir, Srinagar 190011, Jammu and Kashmir, India. syed.mudassar@skims.ac.in

Abstract

BACKGROUND

Connective tissue growth factor (CTGF) is a mediator of transforming growth factor-beta signaling and plays a key role in connective tissue remodeling, inflammatory processes and fibrosis in various illnesses including cancer.

AIM

To investigate the role of CTGF in colorectal cancer (CRC) progression and to compare the CTGF expression with different clinicopathological parameters.

METHODS

Real-time polymerase chain reaction, immunohistochemistry and Western blotting was performed to evaluate the CTGF expression and the results were statistically analyzed against the clinicopathological variables of patient data using STATA software version 16.

RESULTS

and proofreading.

Institutional review board statement: The study was reviewed and approved by the Institutional Ethics committee (IEC), Sher-I-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, India.

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CTGF expression levels in tumor specimens were significantly higher than their paired normal specimens. The higher protein expression levels showed a significant association with smoking, staging, tumor grade, invasion depth, necrosis of tumor tissue, and both lymphovascular and perineural invasion. As *per* the cox regression model and classification tree analysis, tumor-node-metastasis stage and perineural invasion were important predictors for CTGF expression and prognosis of CRC patients. Survival analysis indicated that CTGF overexpression was associated with poorer overall and disease-free survival.

CONCLUSION
Expression of CTGF was increased in CRC and was linked with poor overall and disease-free survival of CRC patients. These findings support prior observations and thus CTGF may be a possible prognostic marker in CRC.

Key Words: Connective tissue growth factor; Quantitative real time polymerase chain reaction; Immunohistochemistry; Western blotting; Colorectal cancer

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Core Tip: This study demonstrates that connective tissue growth factor is overexpressed in colorectal carcinoma and is predominantly localized in the cytoplasm of the cell. The expression pattern of this gene showed a substantial association with aggressive phenotypes of colorectal cancer like late-stage tumor and lymph node metastasis. It also showed a significant correlation with the overall and disease-free survival of colorectal cancer patients, hence could act as a predictive biomarker for diagnostics and prognostics of colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) ranks 3rd worldwide in terms of prevalence and is the second most common type of cancer in terms of mortality, with bowel cancer being the fourth most common cancer and rectal cancer being the eighth most common cancer in world. In 2020, more than 1.9 million new CRC cases including the anus and 935000 deaths were expected, accounting for about one in every ten cases and deaths[1]. Transitioned countries have a four-fold higher incidence rate than transitional countries, but there is less difference in death rates due to the higher fatality rate in transitioning countries[2]. However, most of the cases and deaths are attributed to modifiable risk factors, such as smoking, certain types of diets, high alcohol consumption, sedentary lifestyle, and being over-weight or obese, and thus potentially evadable. Other clinicopathological characteristics like age, family history of CRC or personal history of inflammatory bowel disease or colon polyps and many other inherent risk factors that cannot be modified have progressively been recognized as contributing to a more individual prognosis prediction in CRC[3,4]. CRC is a disease of modernity and heterogeneity: It has the highest rates of incidence and almost 60% of cases are encountered in developed countries. In India, the age-standardized average for CRC is poor, at 7.2 *per* 100000 for men and 5.1 *per* 100000 for women[3].

Connective tissue growth factor (CTGF) or CCN-family protein 2 (CCN2), is a member of the CCN family. The acronym CCN was assigned to this family after the initials of the first three proteins which include cysteine rich-61 (Cyr61/CCN1), connective tissue growth factor (CTGF/CCN2) and nephroblastoma overexpressed (Nov/CCN3)[5]. WNT1 inducible signaling pathway proteins WISP1/CCN4, WISP2/CCN5 and WISP3/CCN6 are other members of this family[6]. These proteins manifest roles in cellular mechanisms like cell proliferation, apoptosis, cell-cell

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adhesion, ion-transport, extracellular matrix production, growth arrest, migration in numerous cell types, chemotaxis and motility[7]. CTGF is also related to growth of bone, cartilage, angiogenesis and is associated with a number of biological response modifiers[8,9]. CTGF expression has been linked to a variety of disorders, including diabetic nephropathy, arthritis, cardiovascular disease, and retinopathy as well as many types of malignancies[10-14]. CTGF shows an aberrant expression in variety of cancers like pancreatic cancer cells[12], breast[15], esophageal cancers[11], in gliomas [10] and invasive melanomas[16]. Expression studies of CTGF in CRC have been reported by some groups[17,18], but no such study has been conducted in our population to date. The Kashmir valley, located in India's extreme north, is very unique in its climate, diet and geology. CRC is the fourth most prevalent kind of cancer in the Kashmir valley, after stomach, esophageal, and lung with a significant increase in a number of cases[19]. To further explore the potential role of CTGF in CRC, we looked at its expression and localization and compared the findings to critical clinicopathological parameters like tumor grade, tumor-node-metastasis (TNM) stage, Duke stage, lymph node metastasis, lymphovascular invasion (LVI), perineural invasion (PNI) and so on. We also performed a survival study analysis on the CRC patients to see whether CTGF has a prognostic role in the disease. In patients with CRC, TNM staging remains the most important prognostic predictor. The systemic spread of tumor cells *via* metastasis is the major factor that leads to cancer recurrence and prognosis. As a result, including LVI and PNI into the present TNM staging method may provide a more accurate indication of patient prognosis in any stage of CRC, allowing for more effective adjuvant therapy planning and patient follow-up.

MATERIALS AND METHODS

Patient specimens

Seventy-one (n = 71) histopathological confirmed human CRC tissues along with adjacent normal tissues (study controls) were taken for the study, which included 38 males and 33 females. The specimens were taken from the subjects who underwent primary surgical resection for CRC at the Department of General and Minimal invasive Surgery, Sher-i-Kashmir Institute of Medical Science (SKIMS) Srinagar. Patients who received neoadjuvant or chemoradiotherapy were excluded from our study. Specimens were preserved in RNA Later (Sigma-Aldrich Burlington, MA) for RNA extraction and held in -80 ℃ till further processing. For immunohistochemistry (IHC), formalin fixed and paraffin embedded blocks of the same specimens and their adjacent normal blocks were collected from the Department of Pathology, SKIMS. Both the samples and blocks were taken in accordance with SKIMS Research Ethics Committee's approved protocol and with the patient's written consent.

RNA isolation and cDNA synthesis and real-time polymerase chain reaction

Total RNA was extracted by the Trizol method (Invitrogen Waltham, MA, United States)[20]. The absorbance at A260/280 of 1.8-2 was considered "pure" for RNA. After DNase-I (Qiagen) treatment, as *per* the manufacturer's instructions, complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (#K1622; Thermo Scientific Ltd, Waltham, MA, United States). In a final volume of 20 µL, 1g RNA was reverse transcribed using AMV Reverse Transcriptase and random hexamers. The cyclic conditions were 5 min at 25 ℃, then 60 min at 42 ℃ and finally 5 min at 70 ℃. Quantitative real time polymerase chain reaction (qRT-PCR) was done using SYBR Green Master Mix (Thermo Fisher Scientific Ltd) in Piko-Real PCR machine (Thermo). Experiments were carried out in triplicates with effects of each being standardized against the housekeeping gene GAPDH. The following primers were used; CTGF-fp: 5'CTCCTGCAGGCTAGAGAAGC3'; rp: 5'GATGCACTTTTTGCCCTTCTT3'. GAPDH-fp: 5'CACCACCAACTGCTTAG3'; rp: 5'CTTCACCACCTTCTTGATG'. The Cycle Threshold (Ct) was used to describe the expression of CTGF messenger RNA (mRNA). The relative expression levels were determined using Livak and Schmittgen's $2^{-\Delta\Delta ct}$ method[21].

The mean Ct values for the gene of interest (*CTGF*) and the control gene (*GAPDH*) were estimated. ΔCt was calculated by subtracting the average of triplicate CTGF Ct values from the average of triplicate GAPDH Ct values. The ΔΔCt stood for the difference between the paired tissues samples, which was determined using the formula ΔΔCt=ΔCt of tumor-ΔCt of normal. The fold-change in tumor levels relative to surrounding normals was estimated as $2^{-\Delta\Delta Ct}$. Denaturation at 95 ℃ for 7 min, 40 cycles of denaturation; 95 ℃ for 5 min, annealing; 59 ℃ for 50s and extension; 60 ℃ for 30s

were used in the qRT-PCR reaction. There was no non-specific product formation according to the melt curve review.

Protein extraction and Western blot assay

About 50-100 mg tissue specimens were washed thrice with icy-cold phosphate-saline buffer in a centrifuge at 3567g for 5-min. The Tissue sections were then homogenized in RIPA Lysis buffer (Cat#20-188; Lot#3283787), to which 1 mmol/L sodium fluoride, 1 mmol/L PMSF and protease inhibitor cocktail 10 µL *per* 1 mL of lysis buffer were added immediately prior to use. After lysis samples were vortexed for 15 s and then incubated on ice for 40 min. Centrifugation at 73 g for 20 min was done and the supernatant containing extracted protein was collected, the protein concentration was measured spectrophotometrically at 595 nm by using Bradford's assay. Protein extract was run on a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel after being preheated at 95 °C for 5 min in reducing SDS sample buffer containing 50 mmol/L TrisHCl (pH6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mmol/L mercaptoethanol. The proteins isolated were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, United States) by semidry transfer method following the manufacturer's instructions (Hoefer) after gel electrophoresis. The PVDF membrane was processed according to a standardized protocol for immune detection. Rabbit polyclonal antibody anti-CTGF was used at 1:1000 dilution (ab5097; Abcam, Cambridge, United Kingdom). Rabbit monoclonal antibody against beta-actin was employed at 1:1000 dilution (Cell Signaling Technology; Danvers, MA, United States). The HRP-tagged secondary antibody (#70749; Cell Signaling Technology) was used for the detection of protein bands. Beta-actin served as a loading control. The blot was detected by chemiluminescence with Super Signal West Femto Maximum Sensitivity Substrate. (Cat No. 34095). Image J software version 15.3a was used to assess the densiometry of the blots to measure the quantity of proteins present.

Immunohistochemical analysis

For immunohistochemical analysis of CRC tissues, the formalin-fixed and paraffin embedded representative blocks were used. A manual microtome (Leica Biosystems, Mumbai, Maharashtra, India) was used to slice 5-µm thick tissue sections from paraffin blocks and mount on charged poly-L-lysin coated glass slides (LOT #180310 Bio-Optica Milano S.p.a *via* San Faustino, 58 20134 Milan-Italy). The sections were deparaffinized using xylene and ethanol, and then they were rehydrated in a graded series of ethanol (100%, 95%, 90%, 80% and 70%), followed by microwave/pressure cooker antigen retrieval. Rehydration of sections was done using distilled water. Hydrogen peroxide blocking solution (Biocare Medical, Condord, CA, United States) was added after rehydration to cover the whole section which acts as an inhibitor of endogenous peroxidase activity. After that was done the slides were incubated for 15 min while keeping the slides hydrated and letting them dry. Slides were washed two to three times with a phosphate buffer saline (PBS) (pH = 7.4). Tumor sections were then treated with 10 mmol/L citrate buffer (pH 6.0) at 95 °C to retrieve antigens. After which the slides were washed three times with PBS. To block non-specific background staining, the sections were covered with protein blocking solution (#BS966G Biocare Medical) which was followed by a 15 minute incubation. Washings were done with PBST (1 × PBS with Tween-20). Slide edges were dried and sections were outlined with boundary by PAP pen (Abcam). Primary antibody anti-CTGF (1:200; Abcam) was put on the whole sections and kept in overnight incubation. Next day the slides were washed thrice using PBS. Secondary antibody coated to goat anti-rabbit HRP-conjugated secondary antibody (#M2U522G; MACH 2 Universal HRP-Polymer Detection Kit) was put on the sections which were then kept in incubation for 15 min. PBST washings were done four times and later stained with HRP/DAB detection IHC kit (#BDB2004H; Biocare Medical). To prepare DAB solution, 30 µL of DAB chromogen (#BDB900C; Biocare Medical) was mixed with 1.5 mL of DAB substrate (#DS900H; Biocare Medical), which was then applied to the sections. The slides were again washed four times with PBST. The slides were immersed in distilled water, counterstained with hematoxylin and thereafter, dehydrated in xylene, mounted with DPX and covered by cover slips. Visualization was done by a light microscope (1 × 81; Olympus, 1 × 81; Tokyo, Japan). Sections from liver carcinoma were used as positive controls. For negative controls, PBS was used instead of primary antibody to tissue sections.

Statistical analysis

For statistical analysis, independent t test and logistic regression was used to calculate

the odds ratio (OR) and 95% confidence interval (95%CI). P value < 0.05 was considered as statistically significant. Analysis of the survival data was done by the Kaplan–Meier method. Kaplan–Meier curves were compared by a log-rank test. Regression analysis was utilized using an extended Cox regression model. All the statistical analysis was done using STATA software, version 16 (STATA 16 Corp., College Station, TX, United States).

Ethical clearance

A written consent was taken from all the patients prior to surgery and they were also apprised about the ongoing study which was approved by Ethical Clearance Committee of SKIMS (SIMS 1131/IEC-SKIMS/2018-330).

Follow-up

Patients were contacted by phone for follow-up. The deadline for contacting the patients was May 2021. The survival intervals were calculated from both the date of diagnosis as well as from the date of surgery to the last follow-up.

RESULTS

Patient characteristics

Seventy-one human histopathological confirmed CRC tissues and paired normal adjacent tissue specimens from patients who went through primary surgical resection for CRC with no chemo/radiotherapy received were used to assess the CTGF expression. Demographic and clinicopathological variables for this study are outlined in Table 1. The cases included 38 (53.52%) males and 33(46.47%) females. In all, 50 of 71 (70.42%) subjects were greater or equal to 50 years and 21 of 71 (29.71%) were less than 50 years having a mean age of 56.04 ± 13.48.

CTGF mRNA expression in CRC

We used qRT-PCR to analyze the expression of CTGF at mRNA level in CRC tissues and the adjacent normal tissues (n = 71). Overexpression of CTGF was detected in 80.28% (57/71) tumor tissues as compared to adjacent normal tissues. The average fold change of CTGF expression in CRC tissues in comparison to normal adjacent tissues was 2.49 ± 1.59. Figure 1 depicts the dot blots of CTGF mRNA indicating ΔCt values. To ensure accuracy, melt curve analysis was performed on every experiment that yielded zero nonspecific results.

CTGF protein expression and localization in CRC

CTGF protein localization and expression pattern was analyzed by IHC in 71 histopathological confirmed CRC specimens and their adjacent normal tissues. In CRC tissues, CTGF immunoreactivity showed a varying intensity of staining with most remarkable staining in cytoplasm of the tumor cells (Figure 2). A scoring system was set for evaluation of CTGF immunoreactivity as 0 (negative staining); +1(less than 5% of the tumor cells stained/Weak staining); +2 (less than 50% tumor cells stained/Moderate staining); and +3 (more than 50% tumor cells stained/Strong staining). Overexpression was said to be intense when cytoplasm stained more than three times in tumor compared to normal adjacent tissues. IHC Profiler plugin ImageJ software (ImageJ version 15.3a) was used for quantification of staining intensity. Out of 71 CRC cases, a total of 44/71 (61.97%) cases showed high expression compared to their normal adjacent tissues. Among all, 26 of 71 (36.61%) displayed a strong (+3) cytoplasmic staining intensity, 18 of 71 (25.35%) showed moderate (+2), 25 of 71 (35.21%) showed weak (+1) intensity and 2 of 71 (2.82%) CRC cases showed negative scoring intensity. The prevalence of high CTGF expression among the 4 TNM stages was 28%, 72%, 88.89% and 100% for Stage I, II, III, and IV respectively among all 71 CRC cases. Among the 44 cases which showed overexpression of CTGF, the stage wise contribution was 15.91% (7/44) in stage I, 40.91% (18/44) in stage II, 36.36% (16) in stage III and 6.82% (3/44) in stage IV disease (P < 0.001; Chi^2 = 20.7).

In order to confirm the results of immunohistochemical staining, western blot analysis was performed to evaluate the CTGF protein levels in the same patient specimens and their adjacent normal tissues. Densitometric image analysis was done by ImageJ version 1.53a software to assess the amount of protein present in the sample. CTGF protein expression was considered high in CRC tumor samples when the level was more than one-fold than that in adjacent normal tissues. Among the 71

Table 1 Clinicoepidemological and clinicopathological parameters of study population (*n* = 71)

Characteristics	*n* (%)
Age in years	
< 50	21 (29.58)
≥ 50	50 (70.42)
Gender	
Male	38 (53.52)
Female	33 (46.48)
Dwelling	
Rural	47 (66.20)
Urban	24 (33.80)
Social class	22 (30.99)
Low	
Middle and high	49 (69.01)
Family history	
Yes	20 (28.17)
No	51 (71.83)
Smoking status	
Yes	40 (56.34)
No	31(43.66)
Lifestyle	
Active	31 (43.66)
Sedentary	40 (56.34)
Salt tea intake	
Yes	65 (91.55)
No	06 (8.45)
Red meat consumption	
Yes	59 (83.10)
No	12 (16.90)
Sundried vegetables	
Yes	48 (67.61)
No	23 (32.39)
Source of drinking water	
Tap water (R)	46 (64.79)
Tap water (L)	07 (9.86)
Others[1]	18 (25.35)
Pickles	
Yes	41 (57.75)
No	30 (42.25)
Pesticide exposure	
Yes	33 (46.48)
No	38 (53.52)
Junk food consumption	

Yes	05 (7.04)
No	66 (92.96)
Site of tumor	
Colon	36 (50.70)
Rectum	20 (28.17)
Rectosigmoid	15 (21.13)
Tumor differentiation	
Well	18 (25.35)
Moderate	46 (64.79)
Poor	07 (9.86)
Invasion depth	
T1	08 (11.27)
T2	22 (30.99)
T3	31 (43.66)
T4	10 (14.08)
T1 + T2	30 (42.25)
T3 + T4	41 (57.75)
TNM staging	
I	25 (35.21)
II	25 (35.21)
III	18 (25.35)
IV	03 (4.22)
I + II	50 (70.42)
III + IV	21 (29.58)
Tumor grade	
1	18 (25.35)
2	46 (64.79)
3	07 (9.86)
DUKE stage	
A	05 (7.04)
B	44 (61.97)
C	22 (30.99)
Node status	
0	51 (71.83)
1 and 2	20 (28.17)
LVI	
Present	54 (76.06)
Absent	17 (23.94)
PNI	
Present	15 (21.12)
Absent	56 (78.87)
TALNR	
Present	62 (87.32)

Absent	09 (12.68)
Necrosis seen	
Yes	18 (25.35)
No	53 (74.65)
Recurrence	
Yes	12 (16.90)
No	59 (83.10)
Vital status	
Alive	66 (92.96)
Dead	05 (7.04)

[1]Others: Spring, Well, Pond.

N: Total Number of samples; R: River water through Tap; L: Lake water through Tap; TNM: Tumor-node-metastasis; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion.

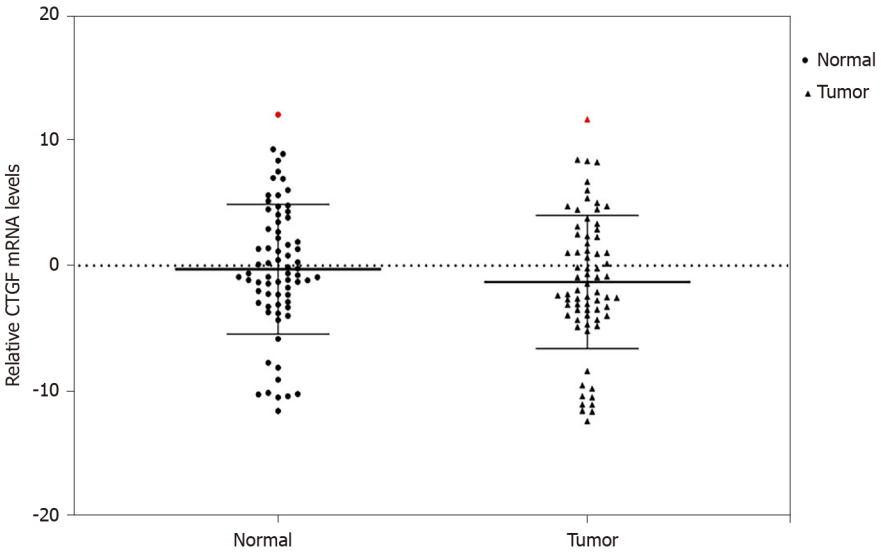


Figure 1 Quantitative real-time polymerase chain reaction analysis of connective tissue growth factor mRNA across the colorectal cancer tissues and their adjacent normals (*n* = 71). mRNA: Messenger RNA; CTGF: Connective tissue growth factor.

CRC cases, 60.56% (43/71) exhibited increased CTGF protein expression in tumor tissues as compared to adjacent normals. Figure 3A shows the representative blot of CTGF protein expression upregulated in CRC tumor tissues and Figure 3B depicts the corresponding bar chart of densiometric values of CTGF protein expression. The CRC samples which showed protein overexpression had also increased mRNA expression. Figure 4 depicts the average fold change in CTGF overexpression in all 71 CRC tumors relative to adjacent normal.

The correlation between real time PCR, western blotting and immunohistochemical analysis was high and significant (P = 0.0001).

Correlation of CTGF with various clinicopathological factors

To better understand the clinical significance of CTGF expression in CRC, expression of CTGF was correlated with a series of clinicopathological parameters. The overexpression of CTGF was associated with smoking, tumor differentiation, invasion depth, TNM stage, tumor grade, Duke staging both at mRNA level as well as at protein level. Node status, lymphovascular, PNI and necrosis of tumor tissues was also significantly associated with the overexpression of CTGF (P < 0.001). Gender was associated with mRNA expression of CTGF (P = 0.037), but not with its protein expression. No significant association with other parameters such as age, sex, dwelling, social class, family history, lifestyle, salt tea intake, sundried vegetables, junk food consumption,

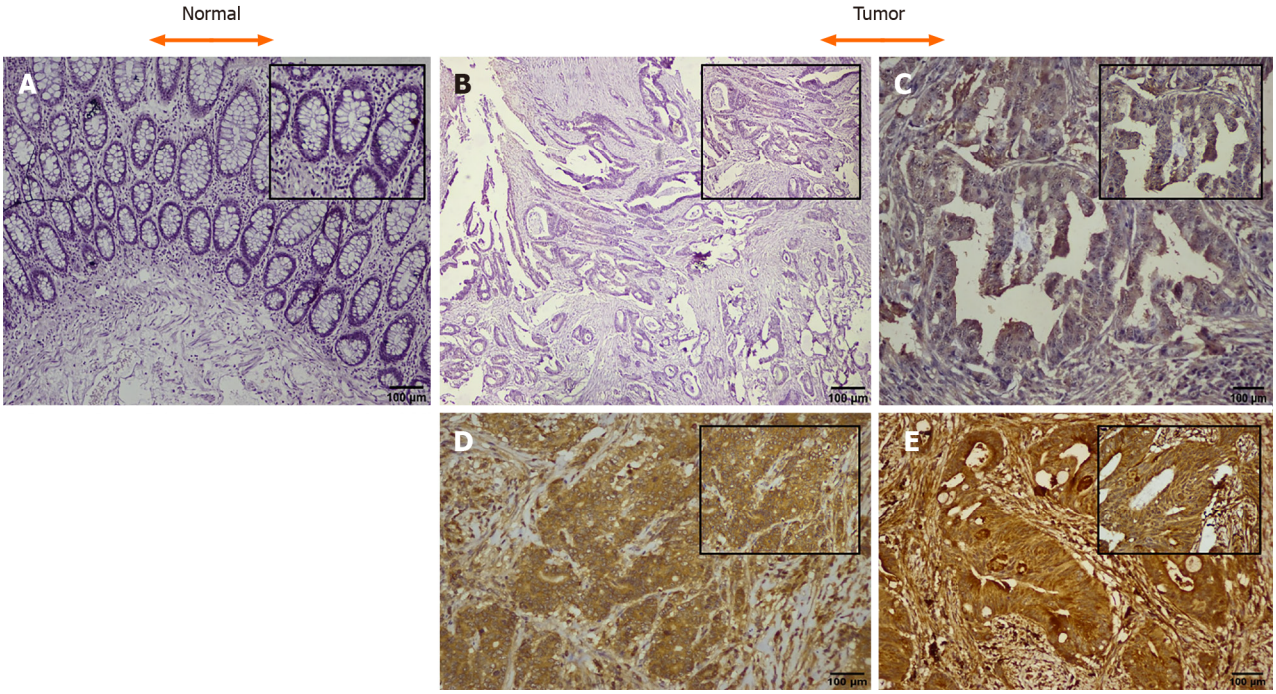


Figure 2 Representative images of immunohistochemical expression of the connective tissue growth factor protein in colorectal cancer and adjacent normal (H & E and DAB Chromogen × 100 insect × 400). A: Negative control; B: Tumor negative staining; C: Tumor + 1, weak staining; D: Tumor + 2, moderate staining; E: Tumor + 3, strong staining. Scale bars: 100 µM.

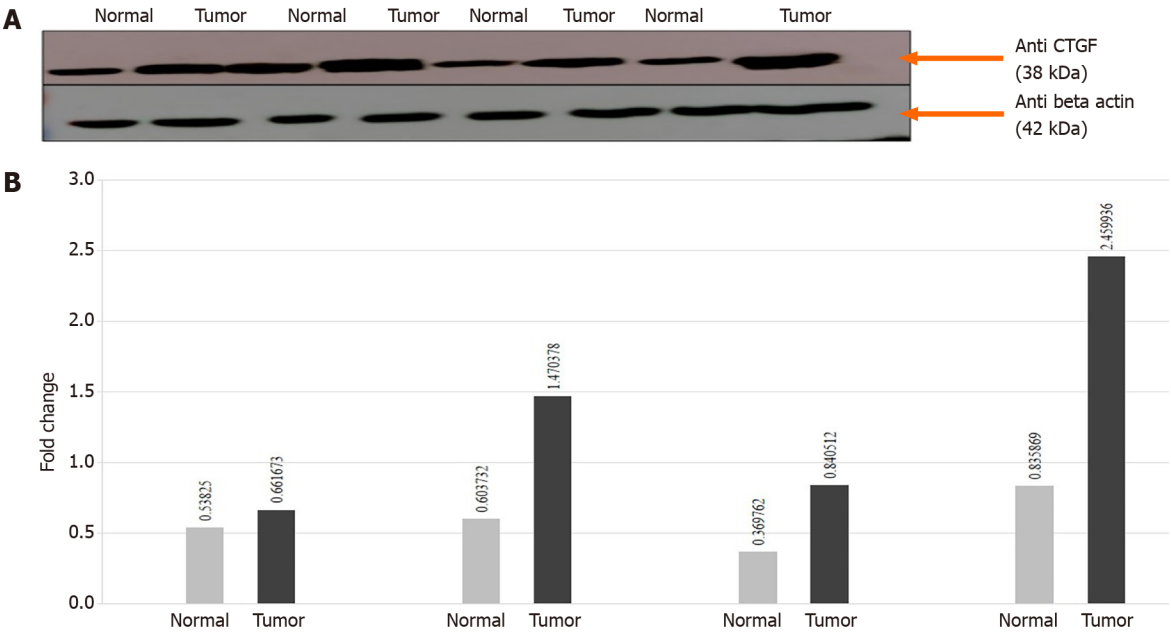


Figure 3 Connective tissue growth factor. A: The connective tissue growth factor (CTGF) is upregulated in human colorectal cancer (CRC) samples. Representative Immunoblot showing expression of tumors and their adjacent normals in CRC. Lane N: Normal; Lane T: Tumor. β-actin is used as a loading control. About 40 µg protein loaded on 12% sodium dodecyl sulphate-Gel transferred to polyvinylidene difluoride membrane, anti-β actin primary Ab (1:1000), anti-CTGF primary Ab (1:500); B: Normalized densitometric values. The experiments were performed in triplicates; mean and standard error was calculated. CTGF: Connective tissue growth factor.

pesticide exposure, use of pickles, source of drinking water and site of tumor was observed at mRNA or protein level. CTGF high expression was also significantly correlated with recurrence of the patients after surgery, but no association was seen with vital status of the CRC patients (Tables 2-4).

Table 2 Clinicopathological relevance of connective tissue growth factor mRNA expression in colorectal cancer patients as determined by quantitative real-time polymerase chain reaction

Characteristics	Normal expression (*n* = 14)	Overexpression (*n* = 57)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	2 (9.52)	19 (90.48)	Referent		
≥ 50	12 (24.00)	38 (76.00)	3.0 (0.6-29.9)	0.16	1.95
Gender					
Male	4 (10.53)	34 (89.47)	Referent		
Female	10 (30.30)	23 (69.70)	3.6 (0.9-17.8)	0.037[a]	4.36
Dwelling					
Rural	11 (23.40)	36 (76.60)	Referent		
Urban	3 (12.50)	21 (87.50)	0.5 (0.1-2.1)	0.27	1.19
Social class					
Low	7 (31.82)	15 (68.18)	Referent		
Middle and high	7 (14.29)	42 (85.71)	0.4 (0.09-1.4)	0.08	2.94
Family history					
Yes	3 (15.00)	17 (85.00)	Referent		
No	11 (21.57)	40 (78.43)	1.5 (0.3-9.7)	0.53	0.39
Smoking status					
Yes	4 (10.00)	36 (90.00)	Referent		
No	10 (32.26)	21 (67.74)	4.3 (1.04-20.68)	0.019[a]	5.46
Lifestyle					
Active	8 (25.81)	23 (74.19)	Referent		
Sedentary	6 (15.00)	34 (85.00)	0.5 (0.1-1.1)	0.25	1.28
Salt tea intake					
Yes	13 (20.00)	52 (80.00)	Referent		
No	1 (16.67)	5 (83.33)	0.8 (0.02-8.1)	0.844	0.03
Red meat consumption					
Yes	13 (22.03)	46 (77.97)	Referent		
No	1 (8.33)	11 (91.67)	0.3 (0.01-2.6)	0.37	1.22
Sundried vegetables					
Yes	9 (18.75)	39 (81.25)	Referent		
No	5 (21.74)	18 (78.26)	1.2 (0.2-4.7)	0.767	0.08
Source of drinking water					
Tap water (R)	12 (26.09)	34 (73.91)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.5 (0.01-5.3)		
Others[1]	1 (5.56)	17 (94.44)	0.4 (0.04-2.4)	0.54	1.21
Pickles					
Yes	8 (19.51)	33 (80.49)	Referent		
No	6 (20.00)	24 (80.00)	1.03 (0.3-3.9)	1.000	0.002
Pesticide exposure					
Yes	7 (21.21)	26 (78.79)	Referent		
No	7 (18.42)	31 (81.58)	0.84 (0.219-3.21)	0.8	0.07

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	14 (21.21)	52 (91.23)	0 (0-3.08)	0.25	1.32
Site of tumor					
Colon	7 (19.44)	29 (80.56)	Referent		
Rectum	6 (30.00)	14 (70.00)	1.8 (0.4-7.45)		
Rectosigmoid	1 (6.67)	14 (93.33)	0.3 (0.01-2.7)	0.23	2.91
Tumor differentiation					
Well	8 (44.44)	10 (55.56)			
Moderate	5 (10.87)	41 (89.13)	Referent		
Poor	1 (14.29)	6 (85.71)	0.15 (0.03-0.7)	0.009[a]	9.35
Invasion depth					
T1	5 (62.50)	3 (37.50)			
T2	7 (31.82)	15 (68.18)	Referent		
T3	2 (6.45)	29 (93.55)	0.3 (0.03-2.0)	0.001[a]	17.21
T4	0 (0)	10 (100)			
T1 + T2	12 (40.00)	18 (60.00)			
T3 + T4	2 (4.88)	39 (95.12)	0.08 (0.008-0.4)	0.000[a]	13.5
TNM staging					
I	11 (44.00)	14 (56.00)	Referent		
II	2 (8.00)	23 (92.00)	0.1 (0.01-0.6)	0.002[a]	14.5
III	1 (5.56)	17 (94.44)	0.07 (0.01-0.7)		
IV	0 (0)	3 (100.00)		0.040[a]	4.21
I + II	13 (26.00)	37 (74.00)			
III + IV	1 (4.76)	20 (95.24)	0.1 (0.01-1.1)		
Tumor grade					
1	8 (44.44)	10 (55.56)			
2	5 (10.87)	41 (89.13)	Referent		
3	1 (14.29)	6 (85.71)	0.1 (0.03-0.7)	0.009[a]	9.35
DUKE stage					
A	2 (2.82)	3 (4.23)			
B	11 (15.49)	33 (46.48)	Referent		
C	1 (1.41)	21 (29.58)	0.5 (0.05-6.8)	0.042[a]	5.20
Node status					
0	13 (25.49)	38 (74.51)	Referent		
1 and 2	1 (5.00)	19 (95.00)	0.1 (0.003-1.2)	0.041[a]	3.81
LVI					
Present	8 (14.81)	46 (85.19)	Referent		
Absent	6 (35.29)	11 (64.71)	3.1 (0.7-12.7)	0.064	3.42
PNI					
Present	0 (0.00)	15 (100)	Referent		
Absent	14 (25.00)	42 (75.00)	0 (0-1.08)	0.031[a]	4.67
TALNR					

Present	11 (17.74)	51 (82.26)	Referent		
Absent	3 (33.33)	6 (66.67)	2.3 (0.32-12.8)	0.272	1.20
Necrosis seen					
Yes	17 (94.44)	1 (5.56)	Referent		
No	40 (75.47)	13 (24.53)	2.7 (0.5-27.7)	0.080	3.10
Recurrence					
Yes	0 (0)	12 (100)	Referent		
No	14 (23.73)	45 (76.27)	0 (0-1.08)	0.060	3.55
Vital status					
Alive	13 (19.70)	53 (80.30)	Referent		
Death	1 (20.00)	4 (80.00)	1.4 (0.02-1.9)	0.85	0.07

[1]Others: Spring, Well, Pond.
[a]$P < 0.05$: Statistical significance.
R: River water through tap; L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

CTGF and CRC prognosis

Kaplan-Meier survival curves from date of diagnosis of the disease and from date of surgery as shown in Figures 5 and 6 revealed that CRC patients with low CTGF protein expression had good overall survival (OS) and disease-free survival (DFS) than those with higher levels of CTGF. The patients had a median survival time of 37 mo from the time that CRC was diagnosed and 28 mo from when the surgery was done and the higher CTGF protein levels were significantly associated with the recurrence of disease (P=0.020). To determine the independent predictive value of CTGF expression, the influence of each clinicopathological parameter and the expression pattern of CTGF on patient OS and DFS was determined using extended Cox-regression model listed in Table 5. Although TNM stage, Duke Stage, Node status, Necrosis and CTGF Expression showed an accord with the DFS, but only TNM stage and PNI were significant independent predictors of OS in the multivariate analysis ($P < 0.05$). A classification tree analysis was also done to explore the relationship of CTGF overexpression with different variables of colorectal tumors (Figure 7). TNM Stage, Duke Stage, Node status and necrosis were important factors for recurrence of the disease and TNM stage with PNI positive tumors were important predictors for CTGF expression in CRC.

DISCUSSION

In the present study, the CTGF mRNA and protein expression in CRC tissues was evaluated and their histopathological confirmed adjacent normals using qRT-PCR, western blotting, and IHC. The results show that 80.28% of CRC tissues at mRNA level and 60.56% of CRC tissues at protein level had overexpression compared to their adjacent normals. CTGF was overexpressed at both the protein and mRNA levels in the majority of samples, showing that CTGF is upregulated at both the transcriptional and translational levels, although some samples showed overexpression at the mRNA level only. CTGF overexpression in CRC has previously been reported in some studies but they did not contain much clinicopathological data of patients. Higher expression levels of CTGF in CRC tissues compared to adjacent normal specimens have been found in cancers of the esophagus, pancreas and gliomas[11,22,23]. In this study, it was observed that CTGF expression was high in 61.97% of tumor sections as compared to their adjacent normals and was predominantly present in cytoplasm of CRC tumor cells. This is analogous to a variety of other tumor forms, such as esophageal and pancreatic cancers[11,22]. Previous CTGF immunohistochemical studies also revealed cytoplasmic or membranous staining[17,18]. Higher levels of CTGF are linked to more advanced disease in esophageal adenocarcinoma and breast cancer[11,24]. CTGF, a well-known transcriptional target of transforming growth factor-beta (TGF-β), was found to have higher mRNA and protein levels in more advanced Duke and TNM

Table 3 Clinicopathological relevance of connective tissue growth factor protein expression in colorectal cancer patients as determined by Western blot analysis

Characteristics	Normal expression (*n* = 28)	Overexpression (*n* = 43)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	6 (28.57)	15 (71.43)	Referent		
≥ 50	22 (44.00)	28 (56.00)	1.9 (0.6-7.1)	0.22	1.47
Gender					
Male	12 (31.58)	26 (68.42)	Referent		
Female	16 (48.48)	17 (51.52)	2.03 (0.7-6.0)	0.15	2.11
Dwelling					
Rural	20 (42.55)	27 (57.45)	Referent		
Urban	8 (33.33)	16 (66.67)	0.7 (0.21-2.1)	0.45	0.57
Social class					
Low	10 (45.45)	12 (54.55)	Referent		
Middle and high	18 (36.73)	31 (63.27)	1.4 (0.5-4.5)	0.57	0.48
Family history					
Yes	7 (35.00)	13 (65.00)	Referent		
No	21 (41.18)	30 (58.82)	0.7 (0.2-2.5)	0.63	0.23
Smoking status					
Yes	11 (27.50)	29 (72.50)	Referent		
No	17 (54.84)	14 (45.16)	3.2 (1.1-9.7)	0.019[a]	5.46
Lifestyle					
Active	12 (38.71)	19 (61.29)	Referent		
Sedentary	16 (40.00)	24 (60.00)	1.05 (0.4-0.1)	0.91	0.01
Salt tea intake					
Yes	25 (38.46)	40 (61.54)	Referent		
No	3 (50)	3 (50)	1.6 (0.2-12.8)	0.58	0.30
Red meat consumption					
Yes	24 (40.68)	35 (59.32)	Referent		
No	4 (33.33)	8 (66.67)	0.7 (0.1-3.1)	0.63	0.22
Sundried vegetables					
Yes	19 (39.58)	29 (60.42)	Referent		
No	9 (39.13)	14 (60.87)	1.0 (0.3-3.0)	0.97	0.01
Source of drinking water					
Tap water (R)	20 (43.48)	26 (56.52)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.21 (0.01-2.3)		
Others[1]	7 (38.89)	11 (61.11)	1.3 (0.3-4.6)	0.34	2.21
Pickles					
Yes	16 (39.02)	25 (60.98)	Referent		
No	12 (40)	18 (60)	1.04 (0.3-3.03)	0.93	0.01
Pesticide exposure					
Yes	15 (45.45)	18 (54.55)	Referent		
No	13 (34.21)	25 (65.79)	0.624 (0.2-1.8)	0.33	0.93

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	28 (42.42)	38 (57.58)	0 (0-1.1)	0.06	3.5
Site of tumor					
Colon	12 (33.33)	24 (66.67)	Referent		
Rectum	12 (60)	8 (40)	3 (0.84-10.89)		
Rectosigmoid	4 (26.67)	11 (73.33)	0.7 (0.13-3.1)	0.077	5.12
Tumor differentiation					
Well	14 (77.78)	4 (22.22)	Referent		
Moderate	13 (28.26)	33 (71.74)	0.1 (0.02-0.5)		
Poor	1 (14.29)	6 (85.71)	0.04 (0.01-0.64)	0.000[a]	15.33
Invasion depth					
T1	7 (87.50)	1 (12.50)			
T2	13 (59.09)	9 (40.91)	Referent	0.000[a]	18.61
T3	7 (22.58)	24 (77.42)	0.04 (0.01-0.44)		
T4	1 (10)	9 (90)			
T1 + T2	20 (66.67)	10 (33.33)			
T3 + T4	8 (19.51)	33 (80.49)	0.12 (0.03-0.4)	0.000[a]	16.12
TNM staging					
I	19 (76.00)	6 (24.00)	Referent		
II	7 (28.00)	18 (72.00)	0.12 (0.02-0.5)	0.000[a]	23.3
III	2 (11.11)	16 (88.89)	0.04 (0.003-0.3)		
IV	0 (0.00)	3 (100)	0 (0-0.5)		
I + II	26 (52.00)	24 (48.00)			
III + IV	2 (9.52)	19 (90.48)	0.1 (0.01-0.5)	0.001[a]	11.2
Tumor grade					
1	14 (77.78)	4 (22.22)	Referent		
2	13 (28.26)	33 (71.74)	0.1 (0.02-0.5)		
3	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	15.33
DUKE stage					
A	4 (80.00)	1 (20.00)	Referent		
B	22 (50.00)	22 (50.00)	4 (0.3-205)		
C	2 (9.09)	20 (90.91)	0.02 (0.01-0.5)	0.001[a]	13.9
Node status					
0	26 (50.98)	25 (49.02)	Referent		
1 and 2	2 (7.14)	18 (41.86)	0.10 (0.01-0.53)	0.001[a]	10.10
LVI					
Present	17 (31.48)	37 (68.52)	Referent		
Absent	11 (64.71)	6 (35.29)	0.2 (0.06-0.90)	0.015[a]	5.97
PNI					
Present	1 (6.67)	14 (93.33)	Referent		
Absent	27 (48.21)	29 (51.79)	0.1 (0.01-0.58)	0.003[a]	8.55
TALNR					

Present	25 (40.32)	37 (59.68)	Referent		
Absent	3 (33.33)	6 (66.67)	0.7 (0.11-3.9)	0.688	0.16
Necrosis seen					
Yes	2 (11.11)	16 (88.89)	Referent		
No	26 (49.06)	27 (50.94)	0.2 (0.03-0.76)	0.004[a]	8.10
Recurrence					
Yes	1 (8.33)	11 (91.67)	Referent		
No	27 (45.76)	32 (54.24)	0.3 (0.05-1.34)	0.016[a]	5.85
Vital status					
Alive	27 (40.91)	39 (59.09)	Referent		
Death	1 (20.00)	4 (80.00)	0.4 (0.007-3.9)	0.36	0.85

[1]Others: Spring, Well, Pond.

[a]$P < 0.05$: Statistical significance.

R: River water through tap, L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

stages of CRC[18,25].

In our study, we correlated the CTGF expression with a number of clinicopathological parameters of patients and we found that CTGF protein overexpression was significantly associated with smoking, tumor differentiation, invasion depth, TNM staging, Duke Staging, Tumor grade and lymph node metastasis ($P < 0.001$). This was also the first study which revealed that the presence of LVI ($P = 0.015$), PNI ($P = 0.016$) and necrosis ($P = 0.008$) in the tumor was significantly associated with the overexpression of CTGF. In consideration with a previous study, smoking is an important factor which might oxidatively activate the latent TGF-β members like CTGF[26]. In another study, rat models were exposed to cigarette smoke, and it was found that CTGF shRNA effectively suppressed the upregulation of CTGF and cyclin D1 expression in narrow, intrapulmonary arteries which indicated that CTGF plays a crucial role in smoke-induced pulmonary vascular remodeling while regulating cyclin D1 expression[27]. In a study, it was found that CTGF mRNA overexpression was significantly associated with Duke Stage which was particularly noticeable in Duke's A and B stage tumors, while as they found noticeable proportions of CTGF protein overexpression in Duke's stage C[18]. In addition to this, CTGF protein expression was higher in more advanced T-stage and lymph node positive CRC samples as *per* the previous studies[25]. The recurrent cases of colon cancer have been recorded in cases where the lesion was initially diagnosed as LVI negative but later revealed to be positive after re-evaluation with additional more deeply cut specimens when the recurrence was discovered. In the current study, LVI was observed in 54/71 (76.00%) CRCs, in which 86.09% had high expression of CTGF and was significantly associated with CTGF overexpression ($P = 0.043$), and the presence increased with tumor stage from 56% in stage I, 80% in stage II, 94.44% in stage III and in all the three cases of stage IV involved in this study. Since our study included only three cases of Stage IV CRC tumors as the samples taken are mostly from the early-stage primary CRC patients, so most of the samples were skipped due to exclusion criteria of chemo/radio therapy which included mostly the late stage or recurrent patients. Most of the CRC patients with stage I or II are treated with surgery, though some stage II patients can be benefited by adjuvant therapy. The preferable method to treat stage III CRC patients is surgery coupled with adjuvant chemotherapy[28,29]. Comparable to other investigations, our findings indicated that LVI was associated with aggressive tumor characteristics such as higher tumor grade and advanced tumor stage ($P = 0.05$). Presence of LVI was a significant prognostic indicator in patients with stage III CRC [30]. PNI was present in 15/71 (21.12%) and the presence increased with the tumor stage from 4% in Stage I, 20% in stage II and 33.33% in Stage III CRC patients and in all 14/15 (93.33%) PNI positive cases showed significant association with CTGF overexpression ($P = 0.020$). The connection of PNI with histological markers associated with tumor growth (lymphatic invasion and venous invasion) suggests that these components may all be involved in the metastatic processes that include vascular emboli, lymphatic invasion, and PNI. It has been shown that PNI status can be used to

Table 4 Clinicopathological relevance of connective tissue growth factor high and low expression status determined by immunohistochemistry in colorectal cancer patients

Characteristics	Low expression (*n* = 27)	High expression (*n* = 44)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	6 (28.57)	15 (71.43)	Referent		
≥ 50	21 (42.00)	29 (58.00)	1.8 (0.5-6.6)	0.28	1.13
Gender					
Male	11 (28.95)	27 (71.05)	Referent		
Female	16 (48.48)	17 (51.52)	2.3 (0.8-6.9)	0.09	2.86
Dwelling					
Rural	19 (40.43)	28 (59.57)	Referent		
Urban	8 (33.33)	16 (66.67)	0.7 (0.2-2.2)	0.56	0.33
Social class					
Low	10 (45.45)	12 (54.55)	Referent		
Middle and high	17 (34.69)	32 (65.31)	1.5 (0.5-5.0)	0.38	0.74
Family history					
Yes	7 (35.00)	13 (65.00)	Referent		
No	20 (39.22)	31 (60.78)	0.8 (0.2-2.7)	0.74	0.10
Smoking status					
Yes	10 (25.00)	30 (75.00)	Referent		
No	17 (54.84)	14 (45.16)	3.6 (1.2-11.3)	0.010[a]	6.61
Lifestyle					
Active	12 (38.71)	19 (61.29)	Referent		
Sedentary	15 (37.50)	25 (62.50)	0.9 (0.3-2.8)	0.92	0.01
Salt tea intake					
Yes	24 (36.92)	41 (63.08)	Referent		
No	3 (50.00)	3 (50.00)	1.7 (0.2-13.6)	0.53	0.41
Red meat consumption					
Yes	23 (38.98)	36 (61.02)	Referent		
No	4 (33.33)	8 (66.67)	0.8 (0.15-3.3)	0.71	0.13
Sundried vegetables					
Yes	19 (39.58)	29 (60.42)	Referent		
No	8 (34.78)	15 (65.22)	0.8 (0.2-2.5)	0.70	0.15
Source of drinking water					
Tap water (R)	20 (43.48)	26 (56.52)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.2 (0.004-2.06)		
Others[1]	6 (33.33)	12 (66.67)	0.6 (0.2-2.3)	0.29	2.41
Pickles					
Yes	16 (59.26)	25 (56.82)	Referent		
No	11 (40.74)	19 (43.18)	0.9 (0.3-2.6)	0.84	0.04
Pesticide exposure					
Yes	15 (55.56)	18 (40.91)	Referent		
No	12 (44.44)	26 (59.09)	0.5 (0.2-1.6)	0.23	1.4

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	27 (40.91)	39 (59.09)	0 (0-1.1)	0.07	3.30
Site of tumor					
Colon	11 (30.56)	25 (69.44)	Referent		
Rectum	12 (60.00)	8 (40.00)	3.4 (0.9-12.5)		
Rectosigmoid	4 (26.67)	11 (73.33)	0.8 (0.1-3.7)	0.06	5.81
Tumor differentiation					
Well	14 (77.78)	4 (22.22)	Referent		
Moderate	12 (26.09)	34 (73.91)	0.1 (0.02-0.4)		
Poor	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	16.52
Invasion depth					
T1	7 (25.93)	1 (2.27)	Referent		
T2	12 (44.44)	10 (22.73)	0.2 (0.003-1.8)	0.001[a]	17.30
T3	7 (25.93)	24 (54.550)	0.04 (0.01-0.4)		
T4	1 (3.70)	9 (20.45)	0.01 (0.01-0.40)		
T1 + T2	19 (70.37)	11 (25.00)			
T3 + T4	8 (29.63)	33 (75.00)	0.14 (0.04-0.4)	0.000**a**	14.11
TNM staging					
I	18 (66.67)	7 (15.91)	Referent		
II	7 (25.93)	18 (40.91)	0.1 (0.03-0.6)	0.000[a]	20.7
III	2 (7.41)	16 (36.36)	0.05 (0.01-0.3)		
IV	0 (0)	3 (6.820)	0 (0-0.6)		
I + II	25 (92.59)	25 (56.82)			
III + IV	2 (7.41)	19 (43.18)	0.12 (0.01-0.5)	0.001[a]	10.31
Tumor grade					
1	14 (77.78)	4 (22.22)	Referent		
2	12 (26.09)	34 (73.91)	0.1 (0.02-0.4)		
3	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	16.52
DUKE stage					
A	4 (80.00)	1 (20.00)	Referent		
B	21 (47.73)	23 (52.27)	0.2 (0.004-2.6)		
C	2 (9.09)	20 (90.91)	0.02 (0.014-0.5)	0.001[a]	13.3
Node status					
0	25 (49.02)	26 (50.98)	Referent		
1 and 2	2 (10.00)	18 (90.00)	0.1 (0.01-0.6)	0.002[a]	9.30
LVI					
Present	17 (31.48)	37 (68.52)	Referent		
Absent	10 (58.82)	7 (41.18)	0.3 (0.1-1.13)	0.043[a]	4.11
PNI					
Present	1 (6.67)	14 (93.33)	Referent		
Absent	26 (46.43)	30 (53.57)	0.1 (0.002-0.6)	0.020[a]	5.42
TALNR					

Present	24 (38.71)	38 (61.29)	Referent		
Absent	3 (33.33)	6 (66.67)	0.8 (0.1-4.1)	0.75	0.09
Necrosis seen					
Yes	24 (47.06)	27 (52.94)	Referent		
No	3 (15.00)	17 (85.00)	0.2 (0.03-0.82)	0.012[a]	6.21
Recurrence					
Yes	1 (8.33)	11 (91.67)	Referent		
No	26 (44.07)	33 (55.93)	0.11 (0.012-0.9)	0.020[a]	5.40
Vital status					
Alive	26 (39.39)	40 (60.61)	Referent		
Death	1 (20.00)	4 (80.00)	0.4 (0.07-4.2)	0.389	0.74

[1]Others: Spring, Well, Pond.
[a]$P < 0.05$: Statistical significance.
R: River water through tap; L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

Table 5 Predictors for recurrence or mortality of colorectal cancer using extended cox-regression analysis model

Characteristics	Disease-free survival			Overall survival		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.66	0.4-6.4	0.45	1.03	0.9-1.1	0.32
Tumor differentiation	1.13	0.5-2.7	0.79	3.1	0.7-14.4	0.15
Tumor grade	1.12	0.4- 2.7	0.79	3.1	0.7-14.4	0.15
Depth invasion	1.6	0.8-3.2	0.18	1.91	0.6-5.7	0.30
TNM stage	2.8	1.38-5.97	0.005[a]	3.7	1.1-12.7	0.043[a]
Duke stage	9.6	2.3-40.5	0.002[a]	3.36	0.6-18.3	0.161
Node status	0.08	0.02-0.37	0.001[a]	0.25	0.04-1.5	0.139
Necrosis	0.26	0.1-0.82	0.022[a]	1.30	0.14-11.6	0.811
LVI	0.25	0.05-1.2	0.090	1.16	3.25e-17	0.08
PNI	0.47	0.14-1.6	0.230	0.16	0.02-0.98	0.041[a]
CTGF expression	0.13	0.01-1.06	0.013[a]	0.39	0.04-3.5	0.33

[a]$P < 0.05$: Statistical significance. HR: Hazard ratio; CI: Confidence interval; TNM: Tumor-node-metastasis; LVI: Lymphovascular invasion; PNI: Perineural invasion; CTGF: Connective tissue growth factor.

aid in identifying the stage II CRC patients for adjuvant treatment chemotherapy[31]. According to certain studies, PNI evaluation utilizing a grading system based on PNI location inside the gut could improve the value of cancer staging[32].

Furthermore, it was observed that a high CTGF expression was associated with high recurrence of the disease, but no significant association was seen with vital status of the patients. The difference in this study may be due to limited sample size and a shorter time period of 4-year survival analysis. Some other studies have looked into the connection between CTGF expression and CRC prognosis, with mixed results. Previously, studies were conducted to examine CTGF expression in primary CRC using IHC and it was observed that elevated CTGF protein levels were linked to a lower probability of peritoneal metastasis and a substantially increased OS and DFS [17,18]. A previous study has shown that CRC patients with low expression levels of CTGF were associated with shorter survival and shorter recurrence time and moreover overexpression of CTGF in human CRC cells showed a decrease in the invasiveness of tumor cells[17]. This seems to be at odds with our findings. The following could be a

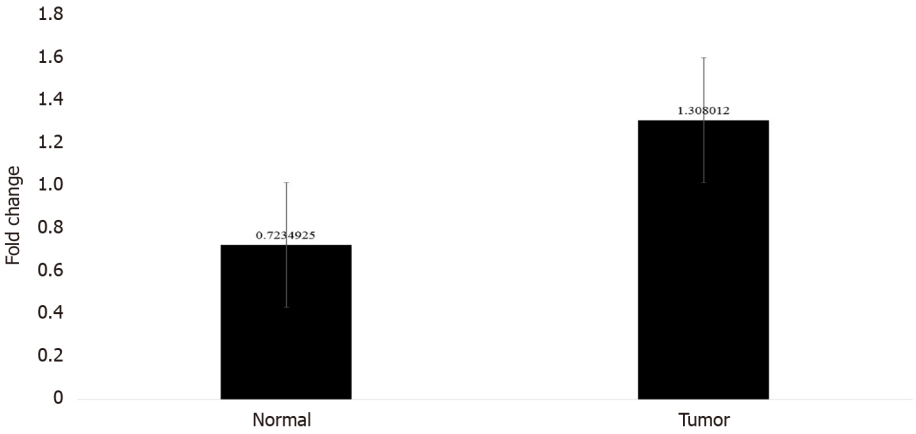


Figure 4 Bar graph showing the average fold change of connective tissue growth factor overexpression in colorectal cancer tumors and their adjacent normal tissue with error bars.

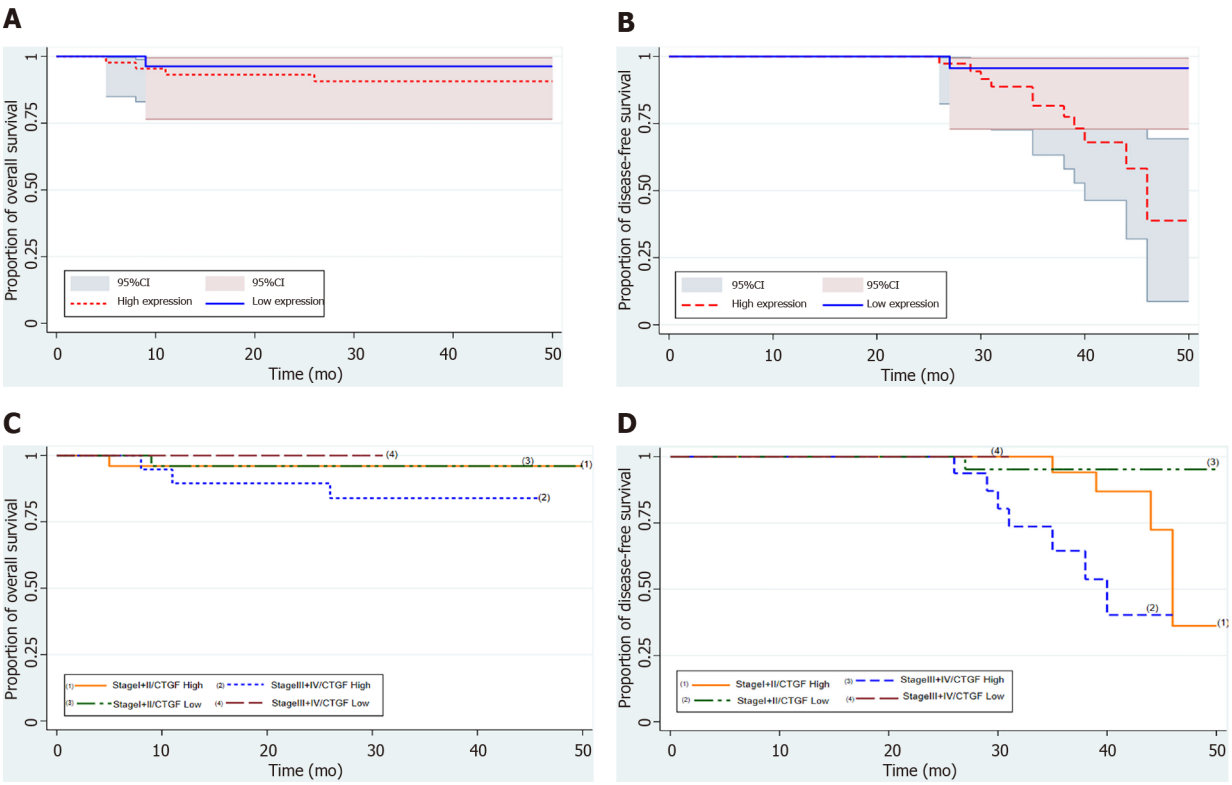


Figure 5 Kaplan-Meier survival analysis of 71 colorectal cancer patients from the time of diagnosis of the disease with connective tissue growth factor protein expression. A: Overall survival (OS) with connective tissue growth factor (CTGF) High and low expression; B: Disease-free survival (DFS) with CTGF High and Low expression; C and D: Depict OS and DFS of colorectal cancer patients respectively stratified as Stage I + II and III + IV.

possible explanation for the aforesaid inconsistency: They compared the CTGF expression with only stage II and III CRC disease, but we stratified the patients broadly into two merged categories of Stage I/II and Stage III/IV. The search for a more accurate mechanism is underway. Previous research has found that tumor stage is the biggest indicator of poor prognosis in people with colon cancer[33]. The results of the present study indicated that high expression levels of CTGF were associated with the TNM Stage which is the most important prognostic factor in CRC. After stratifying our population of 71 CRC patients as *per* TNM stage into I/II and III/IV groups, we found a significant correlation of TNM stage with OS of the patient (P = 0.001; Chi^2 = 17.05) as well as with recurrence of the disease (P = 0.034, Chi^2 = 8.64). Among all, 41.67% of the recurrent cases had Stage I/II CRC disease in which 55.93% had high expression of CTGF protein and about 58.33% recurrent cases had Stage III/IV CRC disease, in which 91.67% of recurrent cases had a high expression of CTGF

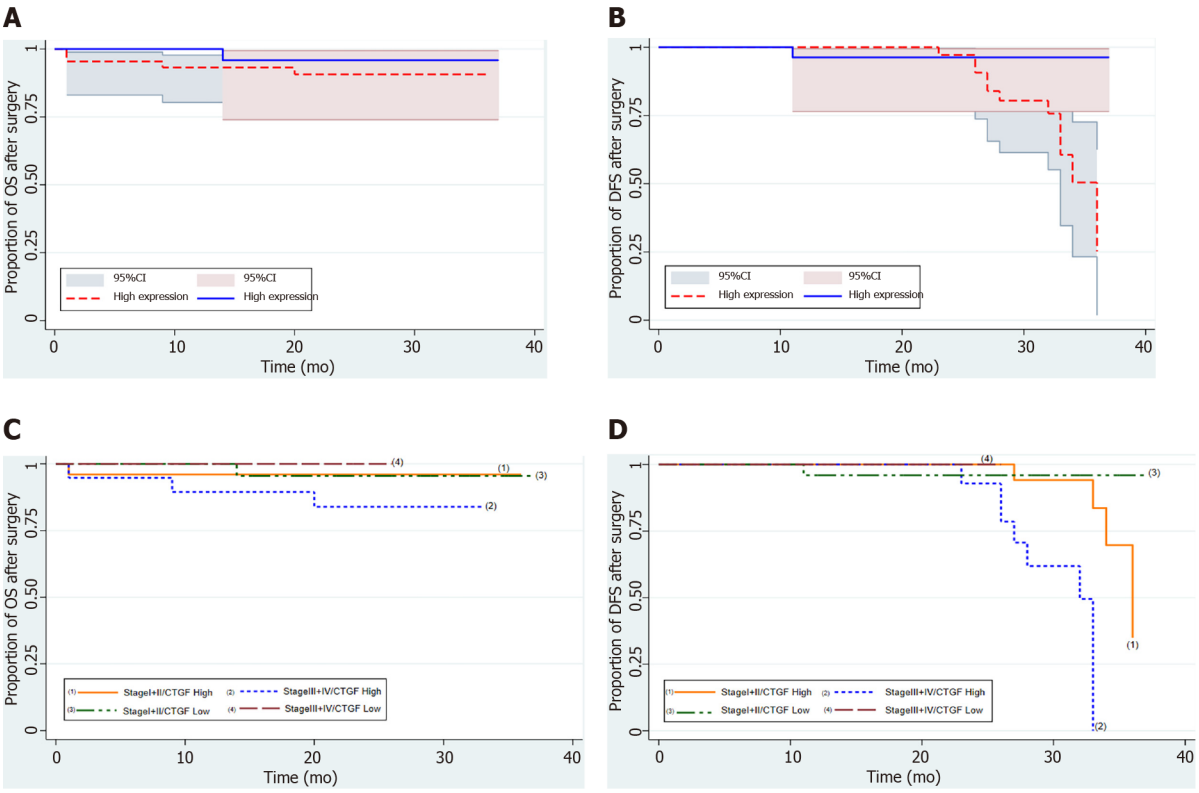


Figure 6 Kaplan-Meier survival analysis of 71 colorectal cancer patients after surgery with connective tissue growth factor protein expression. A and B: Depicts the overall and disease-free survival with connective tissue growth factor High and Low expression respectively; C and D: Shows the overall and disease-free survival of colorectal cancer patients respectively, stratified as Stage I + II and III + IV. CTGF: Connective tissue growth factor; OS: Overall survival; DFS: Disease-free survival.

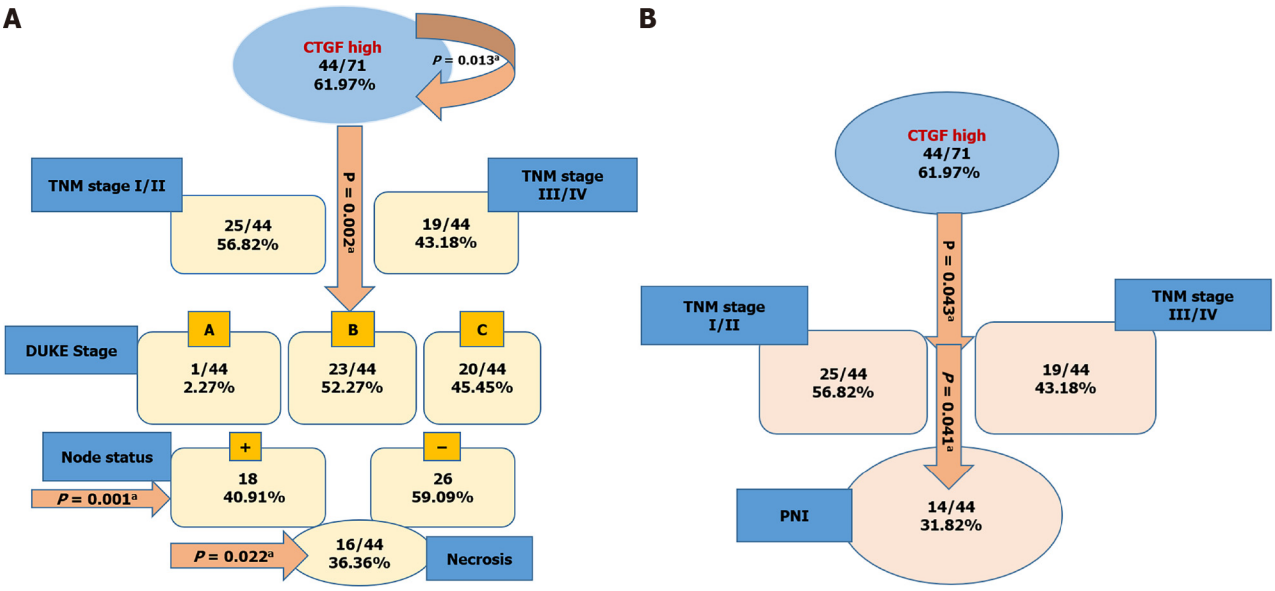


Figure 7 Connective tissue growth factor classification tree analysis for disease-free and overall survival of colorectal cancer patients; the numbers in the circles and boxes shows connective tissue growth factor high expression and percentage of the positive markers *per* total number of cases and arrows show the significant *P* values. A: Tree model of connective tissue growth factor high expression showing that tumor-node-metastasis (TNM) stage, duke stage, lymph node status and Necrosis of tumor tissue were independent predictors for disease-free survival; B: Depicts that TNM Stage and perineural invasion status were independent predictors for overall survival of colorectal cancer patients. $^{a}P < 0.05$. CRC: Colorectal cancer; CTGF: Connective tissue growth factor; PNI: Perineural invasion.

(P = 0.017). In this study, we found that TNM stage was a significant prognostic factor for both OS and DFS of CRC patients, as the stage progresses and CTGF expression increases, the chances of survival decrease in both pre- and postoperative conditions. Hence, proper staging considering LVI and PNI also is conducive to optimal care and has much to do with prognosis of CRC.

CONCLUSION

In conclusion, our results reported strong cytoplasmic localization and overexpression of CTGF in colorectal tumors compared to their adjacent normals. Moreover, the results of this study indicate that the overexpression of CTGF is associated with more intrusive clinicopathological parameters of CRC, suggesting that the expression of CTGF may be important in CRC progression. CTGF expression status could be an independent prognostic factor for CRC patients. LVI and PNI status could be used as complementary factors for TNM staging of CRC. However, because the current study included a limited sample size, more studies with a large number of samples is needed to fully comprehend the mechanism of CTGF induced CRC progression.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is one of the deadliest cancers, having a high death rate. Despite considerable advances in diagnostics and treatments, early detection remains difficult, resulting in a poor prognosis, which is exacerbated by deregulation of critical genes that contribute to disease development.

Research motivation

To investigate the role of connective tissue growth factor (CTGF) in CRC in order to improve the disease diagnosis and prognosis.

Research objectives

To evaluate the expression pattern and localization of CTGF in CRC and correlate it with various clinicopathological variables which might serve as effective preliminary predictive and therapeutic biomarker and aid in future CRC therapies in the Kashmir valley.

Research methods

A total of 71 histopathological confirmed CRC tissues and their adjacent normal specimens were included in this investigation. Real time polymerase chain reaction and western blot were employed to assess CTGF mRNA and protein levels, respectively. Immunohistochemistry was used for confirmation of the CTGF localization. CTGF expression was correlated with various clinicopathological characteristics, and survival analysis was performed on CRC patients to determine whether CTGF plays a predictive role in CRC.

Research results

CTGF expression in tumor samples were significantly higher than in adjacent normal samples. Higher levels of CTGF expression were associated with smoking, staging, tumor grade, invasion depth, necrosis of tumor tissue, lymphovascular and perineural invasion. Tumor-node-metastasis stage and PNI were major predictors of CTGF expression and prognosis in CRC patients, according to the cox regression model and classification tree analysis. CTGF overexpression was linked to poor overall and disease-free survival (DFS), according to a survival analysis.

Research conclusions

CTGF was shown to be overexpressed and cytoplasmic in CRC tumor tissues, according to the findings. Overexpression was related to an aggressive phenotype in CRC patients, as well as poor overall and disease-free survival.

Research perspectives

The study strongly indicates that higher CTGF levels in CRC patients can be employed

as predictive biomarker. Furthermore, CTGF overexpression may serve as a predictive biomarker for patients undergoing a distinct regimen of chemotherapeutic interventions. In order to validate these findings, future investigations with high sample size are needed to completely understand the mechanism of CTGF-induced CRC advancement.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Abnormal liver chemistries as a predictor of COVID-19 severity and clinical outcomes in hospitalized patients

Arunkumar Krishnan, Laura Prichett, Xueting Tao, Saleh A Alqahtani, James P Hamilton, Esteban Mezey, Alexandra T Strauss, Ahyoung Kim, James J Potter, Po-Hung Chen, Tinsay A Woreta

ORCID number: Arunkumar Krishnan 0000-0002-9452-7377; Laura Prichett 0000-0002-6029-6902; Xueting Tao 00-0003-1012-208X; Saleh A Alqahtani 0000-0003-2017-3526; James P Hamilton 0000-0003-3137-7567; Esteban Mezey 00-0001-8217-4466; Alexandra T Strauss 0000-0001-6313-7221; Ahyoung Kim 0000-0003-2633-3738; James J Potter 0000-0002-1558-3945; Po-Hung Chen 0000-0002-9865-1009; Tinsay A Woreta 0000-0001-7292-4518.

Author contributions: Krishnan A and Woreta TA conceptualized and designed the research; Woreta TA supervised the project; Prichett L and Tao XT performed the formal analysis; Krishnan A performed interpretation of data and writing the original draft; Krishnan A, Woreta TA, Alqahtani SA, Hamilton JP, Mezey E and Chen PH performed the review and editing of the draft; All authors revised the manuscript for important intellectual content; All authors approved the final version of the article, including the authorship list

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Arunkumar Krishnan, Saleh A Alqahtani, James P Hamilton, Esteban Mezey, Alexandra T Strauss, Ahyoung Kim, James J Potter, Po-Hung Chen, Tinsay A Woreta, Division of Gastroenterology and Hepatology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, United States

Laura Prichett, Xueting Tao, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21287, United States

Saleh A Alqahtani, Liver Transplant Center, King Faisal Specialist Hospital & Research Center, Riyadh 12713, Saudi Arabia

Corresponding author: Tinsay A Woreta, MD, Assistant Professor, Division of Gastroenterology and Hepatology, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Hal 407, Baltimore, MD 21287, United States. tworeta1@jhmi.edu

Abstract

BACKGROUND

Abnormal liver chemistries are common findings in patients with Coronavirus Disease 2019 (COVID-19). However, the association of these abnormalities with the severity of COVID-19 and clinical outcomes is poorly understood

AIM

We aimed to assess the prevalence of elevated liver chemistries in hospitalized patients with COVID-19 and compare the serum liver chemistries to predict the severity and in-hospital mortality.

METHODS

This retrospective, observational study included 3380 patients with COVID-19 who were hospitalized in the Johns Hopkins Health System (Baltimore, MD, United States). Demographic data, clinical characteristics, laboratory findings, treatment measures, and outcome data were collected. Cox regression modeling was used to explore variables associated with abnormal liver chemistries on admission with disease severity and prognosis

RESULTS

A total of 2698 (70.4%) had abnormal alanine aminotransferase (ALT) at the time of admission. Other more prevalent abnormal liver chemistries were aspartate

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aminotransferase (AST) (44.4%), alkaline phosphatase (ALP) (16.1%), and total bilirubin (T-Bil) (5.9%). Factors associated with liver injury were older age, Asian ethnicity, other race, being overweight, and obesity. Higher ALT, AST, T-Bil, and ALP levels were more commonly associated with disease severity. Multivariable adjusted Cox regression analysis revealed that abnormal AST and T-Bil were associated with the highest mortality risk than other liver injury indicators during hospitalization. Abnormal AST, T-Bil, and ALP were associated with a need for vasopressor drugs, whereas higher levels of AST, T-Bil, and a decreased albumin levels were associated with mechanical ventilation

CONCLUSION
Abnormal liver chemistries are common at the time of hospital admission in COVID-19 patients and can be closely related to the patient's severity and prognosis. Elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies in these patients.

Key Words: Severe acute respiratory syndrome coronavirus 2; Liver injury; Liver tests; Aspartate aminotransferase; Alanine aminotransferase; bilirubin

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Core Tip: Severe acute respiratory syndrome coronavirus-2 primarily infects the respiratory system. However, increasing evidence exists for the direct multiorgan effect. Liver injury in hospitalized patients is associated with a poor prognosis. We investigated whether abnormal liver chemistries in Coronavirus Disease 2019 (COVID-19) hospitalized patients can be of prognostic value. We show that abnormal liver chemistries were commonly observed on hospital admission and are associated with worse outcomes in COVID-19 patients, namely mortality, the need for vasopressor drugs, and mechanical ventilation. In hospitalized COVID-19 patients, elevated liver chemistries, specifically alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin levels, can be used to stratify risk and predict the need for advanced therapies. These results strongly suggest that abnormal liver chemistries at the time of hospital admission are associated with worse outcomes in COVID-19 patients and should be closely followed in admitted patients.

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INTRODUCTION

The Coronavirus Disease 2019 (COVID-19) infection is a global public health crisis that has spread rapidly throughout most of the world and has resulted in over 2 million deaths. Although it is primarily a respiratory disease, increasing evidence indicates that infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the virus that causes COVID-19, can affect multiple organ systems and cause long-term damage[1]. One possible explanation is the route of viral entry into cells *via* the angiotensin-converting enzyme 2 (ACE2) receptor, present on almost all human organs. The liver and the biliary system are no exception, and reports indicate that COVID-19 infection can induce varying degrees of liver injury, ranging from 19%-76% in reported cases[2-4]. The mechanism by which COVID-19 triggers liver injury remains poorly understood.

Direct infection of cholangiocytes *via* ACE2 is postulated as a potential mechanism for intrinsic liver injury from COVID-19[5]. The etiology of the abnormal liver chemistries frequently observed in patients with COVID-19 infection is multifactorial

and associated with major adverse clinical outcomes[6]. The incidence of abnormal liver enzymes significantly increases during the course of the disease, which may indicate the effect of SARS-CoV-2 on the liver or the side effects of medications used to treat the infection[7]. Patients with severe COVID-19 infections have been shown to have higher rates of abnormal liver chemistries[8]. While some studies revealed abnormal liver chemistries are associated with increased disease severity and mortality[9,10], other studies did not find an association with disease progression[11], intensive care unit (ICU) admission[12], or the length of hospital stay[13] and mortality [14]. Thus, study results are inconsistent, with a high degree of heterogeneity.

To address some of these inconsistencies, we examine whether abnormal liver chemistries in COVID-19 hospitalized patients can be of prognostic value. We determined the prevalence of elevated liver chemistries in a large cohort of hospitalized patients with COVID-19 infection and identified whether an independent association exists between abnormal liver chemistries and clinical severity or the risk of in-hospital mortality.

MATERIALS AND METHODS

Study design and participants

In this observational, retrospective cohort study, we analyzed consecutive adult patients (> 18 years of age) who were admitted at the Johns Hopkins Health System (Baltimore, MD, United States) between March 01, 2020, and January 21, 2021, who tested positive for SARS-CoV-2. The diagnosis of COVID-19 was made by at least one positive SARS-CoV-2 real-time PCR test performed on nasopharyngeal swab samples [8]. Only laboratory-confirmed patients were included in this study. This study was approved by the Institutional Review Board (IRB00249001) of the Johns Hopkins University School of Medicine, and the informed consent was waived for a retrospective review of patient charts.

Data collection

We obtained data from the COVID-19 Precision Medicine Analytics Platform Registry (JH-CROWN) database for this cohort study[15], which is a collection of data from the Johns Hopkins electronic health record (Epic) and available for analysis using an electronic database on the Precision Medicine Analystic Platform. Patients without any liver chemistry (n = 1874) results were excluded from the study. Demographic, clinical characteristics, laboratory tests, and treatment were retrieved from the medical records. Furthermore, the clinical outcomes were observed up to January 21, 2021, the final date of follow-up.

Definitions

Elevated liver enzyme levels were defined as patients having alanine aminotransferase (ALT) levels greater than 25 U/L for women and 35 U/L for men, according to the American Association for the Study of Liver Diseases definitions[16]. Other liver chemistry abnormalities were characterized as using the upper limit of the normal range (ULN) for serum levels of aspartate aminotransferase (AST), total bilirubin (T-Bil), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT). Additionally, liver injury was categorized based on the degree of liver enzyme elevation as mild (1-2 times of ULN), moderate (> 2-5 times of ULN), and severe (> 5 times of ULN).

Clinical classification

According to the World Health Organization interim guidance, patients in this study were classified based on COVID-19 disease severity[17]. Cases were defined as either: mild—if patients had mild symptoms of COVID-19 without abnormalities on chest imaging; moderate—if they had respiratory tract symptoms with no obvious hypoxemia and pneumonia manifestation by imaging; severe—if they had any of the following conditions: respiratory rate ≥ 30 breaths/minute; resting fingertip oxygen saturation <90%; a ratio of the arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) < 300mmHg or lung infiltrates critical—if they had respiratory failure requiring mechanical ventilation, or symptoms of shock, or respiratory failure combined with other organ dysfunction requiring intensive care. Identified COVID-19 patients were then stratified into two groups: non-severe (mild and moderate cases) and severe (severe and critical cases) disease, based on the above classification.

Study outcomes

We defined mortality as the primary clinical outcome. Death was assessed at the end of the study period. We also examined the need for vasopressor drugs and mechanical ventilation as the secondary outcomes.

Statistical analysis

Categorical variables were summarized as frequencies (percentages). Chi-squared tests were used to compare categorical variables, and the Mann-Whitney-Wilcoxon test was used for continuous variables. The results are presented as median with interquartile range (IQR). Interaction analyzes were performed as needed. Missing data were not imputed, and only complete cases were included. Patients were considered right-censored if they were discharged from the hospital alive or remained in the hospital at the end of follow-up. We measured time to event in days from the date of hospital admission to the date of in-hospital mortality or hospital discharge alive. Cox regression modeling was used to explore the relationship between abnormal liver biochemistries and mechanical ventilation and risk of death using hazard ratios (HRs) and 95% confidence intervals (CIs). Univariate analyses were used to identify independent risk factors associated with mechanical ventilation and risk of death, and these were ultimately included in a multivariate model with the grade of liver chemistry elevation. Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model. Cox proportional hazards regression models were also used to estimate HRs for the grade of liver chemistry elevation associated with mortality after controlling for the empirical prognostic elements. Kaplan-Meier (KM) method was used to assess differences in mortality by the degree of liver chemistry elevation. The event-free survival rate was estimated using the KM method, and significance was evaluated with the log-rank test. All tests were two-tailed, and statistical significance was determined at P values < 0.05. All statistical data analyzes were conducted with Stata software (version SE16; StataCorp, College Station, TX, United States).

RESULTS

Patients' demographic and clinical characteristics across disease severity groups

A total of 3830 hospitalized patients with confirmed SARS-CoV-2 infection were included in the analysis (Figure 1). Baseline clinical characteristics of the study cohort are summarized in Table 1. Among these patients, 2476 (64.6%) were non-severe cases, and 1354 (35.4%) were classified as severe cases during hospitalization. On hospital admission, abnormal liver chemistries were commonly seen (ALT 70.4%, AST 44.4%, T-Bil 5.9%, and ALP 16.1%) among all patients. The median age was 64.2 years (IQR 49.6-77.3), 51.1% were male, and 34.8% were African Americans. Obesity was present in 1494 (43.7%) of patients, and preexisting liver diseases in 392 (12.2%) patients. Severe disease was associated with older age and male sex. In patients with severe disease, the rate of coexisting diabetes mellitus without and with complications was significantly higher than in the non-severe group. In addition, these patients had higher cardiovascular disease, chronic respiratory disease, kidney disease, and neurological disease as comorbidities (P < 0.001), as well as significantly higher white blood cell and neutrophil counts and C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, ferritin, prothrombin time (PT), international normalization ratio, D-dimer, lactate dehydrogenase (LDH), and cardiac troponin levels (P < 0.001). Levels of absolute lymphocyte, red blood cell, albumin, and total protein were lower (P < 0.001) in patients with severe disease

Prevalence and degree of abnormal liver chemistries according to COVID-19 disease severity

We compared the abnormal liver chemistries at different cut-off values as 1-2 ×, >2-5 ×, and > 5 × ULN, respectively, between the two groups (Table 1). On hospital admission, abnormal liver chemistries were commonly observed, and most patients had mild elevations within 1-2 × ULN. ALT, AST, T-Bil, and ALP levels were higher and more common in patients with severe disease. Overall, 2698 (70.4%) patients had an elevated ALT level, and the median ALT level was 28 U/L (IQR 18-47). A higher proportion of patients with severe disease had elevated ALT compared to the non-severe patients. The median ALT level was 27 U/L in non-severe disease compared to

Table 1 Baseline and clinical characteristics of patients with a positive test for severe acute respiratory syndrome coronavirus-2

Variables	All patients (*n* = 3830)	Non-severe[1] (*n* = 2476)	Severe[1] (*n* = 1354)	*P* value
Age in yr, median (IQR)	64.2 (49.6- 77.3)	62.1 (45.2-76)	67.4 (55.6-79.1)	< 0.001
Sex, *n* (%), *Male*	1959 (51.1)	1179 (47.6)	780 (57.6)	< 0.001
Ethnicity, *n* (%), *Hispanic*	817 (21.5)	565 (23)	252 (18.8)	0.003
Race, *n* (%)				0.12
White	1389 (36.6)	877 (35.7)	512 (38.2)	
Black	1323 (34.8)	848 (34.5)	475 (35.4)	
Asian	203 (5.3)	133 (5.4)	70 (5.2)	
Other	883 (23.2)	600 (24.4)	283 (21.1)	
BMI (kg/m^2), n (%)				0.11
≤ 18.5	794 (23.2)	519 (23.4)	275 (22.8)	
18.5-24.9	98 (2.9)	56 (2.5)	42 (3.5)	
25-29.9	1036 (30.3)	693 (31.3)	343 (28.4)	
≥ 30.0	1494 (43.7)	946 (42.7)	548 (45.4)	
Comorbidities, *n* (%)				
Chronic liver disease	465 (12.1)	295 (11.9)	170 (12.6)	0.56
Cardiovascular disease				
Congestive heart failure	869 (22.7)	395 (16)	474 (35)	< 0.001
HT without complications	2575 (67.2)	1766 (71.3)	717 (53)	< 0.001
HT with complications	1347 (35.2)	710 (28.7)	637 (47)	< 0.001
Diabetes				
Diabetes without complications	1459 (38.1)	856 (34.6)	603 (44.5)	0.017
Diabetes with complications	1270 (33.2)	679 (27.4)	591 (43.6)	< 0.001
Chronic respiratory disease	1065 (27.8)	618 (25)	447 (33)	< 0.001
Chronic neurological disease	1033 (27.0)	569 (23)	464 (34.3)	< 0.001
CKD of any stage	973 (25.4)	491 (19.8)	482 (35.6)	< 0.001
Anemia	1655 (43.2)	906 (36.6)	749 (55.3)	< 0.001
Hypothyroidism	557 (14.5)	330 (13.3)	227 (16.8)	0.004
Malignancies				
Primary cancer	458 (12)	276 (11.1)	182 (13.4)	0.036
Metastatic cancer	277 (7.2)	167 (6.7)	110 (8.1)	0.12
Laboratory findings, median (IQR)				
Liver biochemistries:				
ALT, median (IQR)	28 (18-47)	27 (18-45)	30 (19-49)	0.003
Normal, *n* (%)	1132 (29.6)	852 (34.4)	280 (20.7)	
Abnormal, *n* (%)	2698 (70.4)	1624 (65.6)	1074 (79.3)	< 0.001
1-2 ULN, *n* (%)	1225 (32)	829 (33.5)	396 (29.2)	
> 2-5 ULN, *n* (%)	1009 (26.3)	583 (23.5)	426 (31.5)	
> 5 ULN, *n* (%)	464 (12.1)	212 (8.6)	252 (18.6)	
AST, median (IQR)	36 (25-55)	34 (24-51.5)	42 (29-64)	< 0.001
Normal, *n* (%)	2046 (55.6)	1443 (60.5)	603 (46.4)	
Abnormal, *n* (%)	1637 (44.4)	941 (39.5)	696 (53.6)	< 0.001

1-2 ULN, *n* (%)	1187 (32.2)	704 (29.5)	483 (37.2)	
> 2-5 ULN, *n* (%)	361 (9.8)	194 (8.1)	167 (12.9)	
> 5 ULN, *n* (%)	89 (2.4)	43 (1.8)	46 (3.5)	
T.Bil, median (IQR)	0.5 (0.3-6.1)	0.4 (0.3-6.0)	0.5 (0.4-7.0)	< 0.001
Normal, *n* (%)	3496 (94.1)	2286 (95.3)	1210 (91.7)	
Abnormal, *n* (%)	221 (5.9)	112 (4.7)	109 (8.3)	< 0.001
1-2 ULN, *n* (%)	177 (4.8)	89 (3.7)	88 (6.7)	
> 2-5 ULN, *n* (%)	34 (0.9)	17 (0.7)	17 (1.3)	
> 5 ULN, *n* (%)	10 (0.3)	6 (0.3)	4 (0.3)	
ALP, median (IQR)	78 (61-103)	77 (61-100)	79 (61-108)	0.014
Normal, *n* (%)	3183 (83.9)	2101 (85.6)	1082 (80.7)	
Abnormal, *n* (%)	611 (16.1)	353 (14.4)	258 (19.3)	< 0.001
1-2 ULN, *n* (%)	525 (13.8)	311 (12.7)	214 (16)	
> 2-5 ULN, *n* (%)	78 (2.1)	38 (1.5)	40 (3)	
> 5 ULN, *n* (%)	8 (0.2)	4 (0.2)	4 (0.3)	
GGT, median (IQR)	119 (63-199)	116 (80-161)	144.5 (59-245)	0.54
Serum Albumin, g/dL median (IQR)	3.8 (3.4- 4.1)	3.9 (3.5-4.2)	3.6 (3.1-3.9)	< 0.001
Total protein (g/L), median (IQR)	6.5 (5.9- 7.1)	6.7 (6.1-7.2)	6.3 (5.7-6.9)	< 0.001
Coagulation test: median (IQR)				
PT (s)	11 (10.5-11.9)	10.9 (10.4-11.6)	11.4 (10.8-12.4)	< 0.001
INR	1.07 (1-1.14)	1.05 (1.0-1.1)	1.1 (1.02-1.20)	< 0.001
APTT (s)	26.2 (1.2-32.2)	25.9 (1.1-31)	25.7 (1.3-33.6)	< 0.001
D-Dimer	1.0 (0.57, 2.06)	0.83 (0.5-1.62)	0.4 (0.8-3.0)	< 0.001
Routine blood tests: median (IQR)				
Hemoglobin (g/dL)	11.9 (10.1, 13.3)	12.2 (10.7-13.6)	10.9 (9-12.7)	< 0.001
White blood cell (/mcL)	7.4 (5.1-10.5)	6.8 (4.7-9.3)	9 (6.4-13)	< 0.001
Red blood cells (/mcL)	4.06 (3.34-4.63)	4.19 (3.59-4.70)	3.81 (3.02-4.46)	< 0.001
Platelets (/mcL)	229 (168-309)	223 (166-298)	238 (172-327)	< 0.001
Neutrophils(/mcL)	36 (5.23-73)	36 (4.6-70.7)	38.9 (6.75-77.9)	< 0.001
Lymphocytes (/mcL)	15.4 (9.2-23.6)	17.8 (11.3-26.1)	11.5 (6.3-18.4)	< 0.001
Renal function tests: median (IQR)				
Creatinine (mg/dL)	0.9 (0.7-1.3)	0.85 (0.7-1.1)	1.03 (0.7-1.8)	< 0.001
Blood urea nitrogen, (mmol/L)	17 (11-28)	15 (10-22)	25 (16-42)	< 0.001
Sodium (mEq/L)	138 (136-141)	138 (136-140)	139 (136-143)	< 0.001
Potassium (mEq/L)	4.1 (3.8-4.5)	4.1 (3.8-4.4)	4.2 (3.8-4.6)	< 0.001
Inflammatory markers: median (IQR)				
Interleukin-6 (pg/mL)	37 (14.5-90.8)	24.7 (10.6-52.2)	81.83 (31.2-181)	< 0.001
Ferritin (ng/mL)	587 (265-1090)	482 (207-900)	810 (413.5-1462.5)	< 0.001
C reactive protein (mg/L)	8.4 (3.4-21.4)	6.4 (2.5-15.5)	13.6 (6.6-33.2)	< 0.001
Fibrinogen (mg/dL)	495 (387-622)	475 (374-579)	524 (393-653)	< 0.001
Lactate(mmol/L)	1.4 (1.1-2.0)	1.3 (1.0-1.7)	1.6 (1.2-2.3)	< 0.001
Cardiac markers: median (IQR)				
Cardiac troponin I (ng/L)	0.07 (0.04-0.18)	0.05 (0.03-0.1)	0.09 (0.05-0.27)	< 0.001

Lactate dehydrogenase (U/L)	327 (245-460)	303 (229-411)	385 (290-533)	< 0.001

[1]Based on the World Health Organization disease severity classification.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IQR: Interquartile range; BMI: Body mass index; Fio2: Fraction of inspired oxygen; HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency síndrome; HT: Hypertension; CKD: Chronic kidney disease; ALT: Alanine aminotransferases; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; T-Bil: Total bilirubin; PT: Prothrombin time; INR: International normalized ratio; APTT: Activated partial thromboplastin time; ULN: Upper limit of normal.

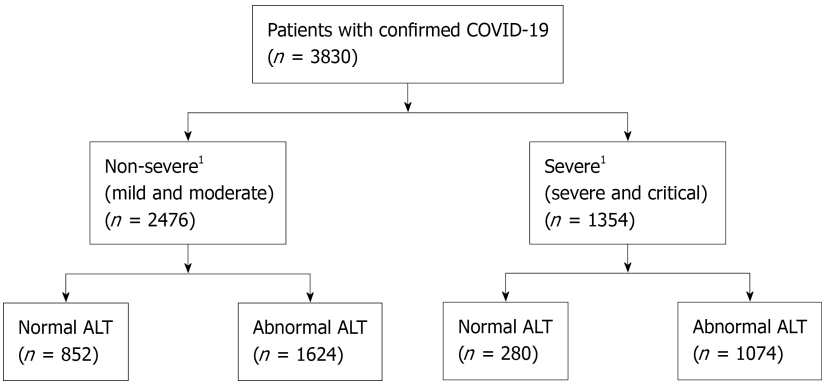


Figure 1 Flow chart of the study. [1]Severity of the diseases is based on the World Health Organization, Classification. COVID-19: Coronavirus Disease 2019 (COVID-19); ALT: Alanine aminotransferase. ALT cut of value was defined as patients having ALT levels greater than 25 U/L for women and 35 U/L for men, according to the AASLD definitions.

30 U/L in severe cases (P = 0.003). In addition, there was a significant difference in the degree of ALT elevation between the two groups (P < 0.001). An elevated ALT at > 2-5 × ULN and > 5 × ULN were significantly more common among patients with severe disease than non-severe. The median AST was 34 U/L (IQR, 24-51.5) in non-severe cases *vs* 42 U/L (IQR, 29-64) in severe cases (P < 0.001). The elevated AST level of 1-2 × ULN (37.2% *vs* 29.5%), > 2-5 × ULN (12.9% *vs* 8.1%) and > 5 × ULN (3.5% *vs* 1.8%) were significantly more common among severe patients compared to non-severe (P < 0.001). Patients with severe COVID-19 also had a higher median T-Bil compared with non-severe patients. However, there was no difference in T-Bil distribution at different levels between the two severities (P = 0.056). Again, in patients with severe disease, the median ALP elevation was higher in the non-severe patients (P = 0.014). However, only four patients in both groups at >5 × ULN had elevated ALP levels. Nevertheless, there was a significant difference in ALP distribution levels between the two severity groups (P < 0.001). Finally, there was no variation in GGT levels at the three thresholds between the two groups (P = 0.540)

Risk factors and predictors of liver injury: Univariate analyses showed that older age, Asian ethnicity, and being overweight were associated with liver injury, whereas other races and obesity were associated with severe liver injury (Figure 2).

In-hospital management and clinical outcomes

In-hospital management and clinical outcomes of patients with COVID-19 infection are shown in Table 2. Patients with severe diseases received more oxygen support and invasive ventilation (P < 0.001). Compared with the non-severe group, patients with the severe disease were more likely to receive antibacterial, antifungal, remdesivir, statins (P < 0.001), antiviral, and NSAID treatment (P = 0.005). The proportion of patients who received azithromycin and dexamethasone treatment was 24.7% and 40.7%, respectively. Moreover, 587 (40.7%) of the severe cases required vasopressor drugs for multiorgan failure (P < 0.001). Severe patients were more likely to develop kidney injury and need renal replacement therapy than non-severe patients. The median length of hospitalization prior to the death was 10.3 d (IQR, 4.5 to 18.8 d). The median length of stay was 6.3 d (IQR, 3.4 to 12.1 d), but patients with severe diseases had a significantly extended hospital stay compared with the non-severe group (median, 4.8 d *vs* 12.3 days, P < 0.001).

Major outcomes

Mortality: Overall, 461 (12.1%) patients died, and all the patients belonged to the

Table 2 In-hospital management and outcomes of patients with a positive test for severe acute respiratory syndrome coronavirus-2

Characteristics	All patients (*n* = 3830)	Non-severe[1] (*n* = 2476)	Severe[1] (*n* = 1354)	*P* value
Respiratory support, *n* (%)				
Non-rebreathing oxygen face mask	3147 (82.2)	1821 (73.5)	1326 (97.9)	< 0.001
High-flow nasal cannula oxygen therapy	843 (22)	18 (0.7)	825 (60.9)	< 0.001
Pharmacological treatment, *n* (%)				
NSAIDs	3303 (86.2)	2107 (85.1)	1196 (88.3)	0.005
Antiviral therapy	192 (5)	106 (4.3)	86 (6.4)	0.005
Antibacterial therapy	2649 (69.2)	1483 (59.9)	1166 (86.1)	< 0.001
Antifungal therapy	234 (6.1)	59 (2.4)	175 (12.9)	< 0.001
Azithromycin	947 (24.7)	530 (21.4)	417 (30.8)	< 0.001
Hydroxychloroquine	423 (11)	239 (9.7)	184 (13.6)	< 0.001
Oseltamivir	10 (0.3)	4 (0.2)	6 (0.4)	0.10
Remdesivir	1303 (34)	741 (29.9)	562 (41.5)	< 0.001
Vitamin D	391 (10.2)	227 (9.2)	164 (12.1)	0.004
Statins	1457 (38)	865 (34.9)	592 (43.7)	< 0.001
ACE inhibitors	378 (9.9)	215 (8.7)	163 (12)	< 0.001
ARB inhibitors	414 (10.8)	246 (9.9)	168 (12.4)	0.018
Immunomodulatory therapy, *n* (%)				
Dexamethasone	1560 (40.7)	881 (35.6)	679 (50.1)	< 0.001
Tocilizumab	95 (2.5)	3 (0.1%)	92 (6.8)	< 0.001
Advanced therapies, *n* (%)				
Vasopressors	587 (15.3)	2 (0.1)	585 (43.2)	< 0.001
Renal replacement therapy/dialysis	188 (4.9)	6 (0.2)	182 (13.4)	< 0.001
Clinical outcome, *n* (%)				
Discharged alive from hospital	3138 (87.2)	2372 (100)	766 (62.4)	< 0.001
Median length of hospital stay (IQR)	6.3 (3.4-12.1)	4.8 (2.8-7.7)	12.3 (7.1-22.3)	< 0.001

[1]Based on the World Health Organization disease severity classification.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus; IQR: Interquartile range; NSAIDs: Nonsteroidal anti-inflammatory drugs; ACE: Angiotensin-converting enzyme; ARB: Angiotensin II receptor blockers.

severe diseases group. 3138 (87.2%) were discharged at the time of data collection for this analysis. In addition, compared to survivors, non-survivors were older and had significantly higher rates of comorbidities (Table 3). In multivariable Cox proportional hazards analysis, increasing age, overweight, obesity, hypertension without complications, chronic neurological disease, and kidney disease were independently associated with an increased risk of in-hospital mortality after adjusting for confounders. Moreover, the results indicated that abnormal AST, T-Bil, ALP, and PT levels were significantly associated with all-cause mortality in all patients with COVID-19, but not the preexisting chronic liver disease, ALT, and albumin levels. Furthermore, a higher state of inflammation was also associated with mortality, with statistically significant increased levels of neutrophil count (P = 0.008), ferritin (P = 0.001), D-Dimer (P = 0.004), CRP, and IL-6 (P < 0.001).

Determining the association of changes in liver chemistries and mortality: The associated distribution of liver chemistries at different levels with in-hospital mortality in patients with COVID-19 was explored using the Cox proportional hazards model (Tables 4 and Figure 3). Unadjusted models showed that a stepwise increase in liver chemistries levels conferred an incremental risk of in-hospital death. Patients with abnormal AST, T-Bil, and ALP levels during hospitalization had a higher mortality

Table 3 Major outcome as the need for mechanical ventilation and mortality among patients with a positive test for severe acute respiratory syndrome coronavirus-2

Liver function test	No mechanical ventilation (*n* = 3205)	Mechanical ventilation (*n* = 625)	*P* value	Survivor (*n* = 3369)	Non-survivors (*n* = 461)	*P* value
Age in yr, median (IQR)	63.8 (48.4-77.7)	66.3 (54.9-75.2)	0.030	62.3 (47.7-74.7)	78.1 (66.9-87.6)	< 0.001
Comorbidities, *n* (%)						
Chronic liver disease	385 (12)	80 (12.8)	0.58	419 (12.4)	46 (10)	0.13
Cardiovascular disease						
Congestive heart failure	662 (20.7)	207 (33.1)	< 0.001	693 (20.6)	176 (38.2)	< 0.001
HT without complications	2112 (65.9)	463 (74.1)	< 0.001	2214 (65.7)	361 (78.3)	< 0.001
HT with complications	1059 (33)	288 (46.1)	< 0.001	1097 (32.6)	250 (54.2)	< 0.001
Diabetes						
Diabetes without complications	1161 (36.2)	298 (47.7)	< 0.001	1269 (37.7)	190 (41.2)	0.14
Diabetes with complications	979 (30.5)	291 (46.6)	< 0.001	1076 (31.9)	194 (42.1)	< 0.001
Chronic respiratory disease	865 (27)	200 (32)	0.011	910 (27)	155 (33.6)	0.03
Chronic neurological disease	810 (25.3)	223 (35.7)	< 0.001	835 (24.8)	198 (43)	< 0.001
CKD of any stage	764 (23.8)	209 (33.4)	< 0.001	787 (23.4)	186 (40.3)	< 0.001
Anemia	1281 (40)	374 (59.8)	< 0.001	1379 (40.9)	276 (59.9)	< 0.001
ALT, median (IQR)	27 (18-46)	33 (21-54)	< 0.001	28 (18-47)	27 (17-45)	0.28
Normal, *n* (%)	1055 (32.9)	77 (12.3)		1011 (30)	121 (26.2)	
1-2 ULN, *n* (%)	1065 (33.2)	160 (25.6)	< 0.001	1090 (32.4)	135 (29.3)	< 0.001
> 2-5 ULN, *n* (%)	786 (24.5)	223 (35.7)		902 (26.8)	107 (23.2)	
> 5 ULN, *n* (%)	299 (9.3)	165 (26.4)		366 (10.9)	98 (21.3)	
AST, median (IQR)	35 (24-54)	44.5 (31-70)	< 0.001	35 (25 -54)	45 (29 -71)	< 0.001
Normal, *n* (%)	1794 (58.3)	252 (41.7)		1856 (57.2)	190 (43.5)	
1-2 ULN, *n* (%)	946 (30.7)	241 (39.9)	< 0.001	1023 (31.5)	164 (37.5)	< 0.001
> 2-5 ULN, *n* (%)	283 (9.2)	78 (12.9)		300 (9.2%)	61 (14.0)	
> 5 ULN, < 0.001 (%)	56 (1.8)	33 (5.5)		67 (2.1%)	22 (5)	
Bilirubin, median (IQR)	0.5 (0.3-0.6)	0.5 (0.4-0.7)	< 0.001	0.5 (0.3-0.6)	0.5 (0.4-0.8)	< 0.001
Normal, *n* (%)	2943 (94.7)	553 (90.8)		3102 (94.9)	394 (88.1)	
1-2 ULN, *n* (%)	133 (4.3)	44 (7.2)	< 0.001	135 (4.1)	42 (9.4)	< 0.001
> 2-5 ULN, *n* (%)	23 (0.7)	11 (1.8)		26 (0.8)	8 (1.8)	
> 5 ULN, *n* (%)	9 (0.3)	1 (0.2)		7 (0.2)	3 (0.7)	
ALP, median (IQR))	77 (61-101)	80 (62-110)	0.062	77 (61-100)	87 (64-118)	< 0.001
Normal, *n* (%)	2686 (84.5)	497 (80.7)		2837 (85)	346 (75.7)	
1-2 ULN, *n* (%)	420 (13.2)	105 (17)	0.091	435 (13)	90 (19.7)	< 0.001
> 2-5 ULN, *n* (%)	65 (2)	13 (2.1)		58 (1.7)	20 (4.4)	
> 5 ULN, *n* (%)	7 (0.2)	1 (0.2)		7 (0.2)	1 (0.2)	
GGT, median (IQR)	116 (56-199)	144.5 (106-235.5)	0.483	117 (56-199)	180 (116.5-447.5)	0.28

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IQR: Interquartile range; HT: Hypertension; CKD: Chronic kidney disease; ALT: Alanine

aminotransferases; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; T-Bil: Total bilirubin; ULN: Upper limit of normal.

Table 4 Multivariable Cox proportional hazards model for outcomes among hospitalized patients with a positive test for severe acute respiratory syndrome coronavirus-2

Clinical predictors	Mechanical ventilation[1]		Mortality[1]	
	Multivariable HR (95%CI)	*P* Value	Multivariable HR (95%CI)	*P* Value
Age	1.00 (0.99-1.01)	0.553	1.04 (1.03-1.05)	< 0.001
Male Gender	1.19 (0.99-1.43)	0.067	1.11 (0.90-1.37)	0.338
Overweight	0.93 (0.73-1.19)	0.577	0.75 (0.59-0.97)	0.030
Obesity	0.94 (0.74-1.19)	0.609	0.58 (0.44-0.77)	< 0.001
Liver diseases	0.84 (0.65-1.09)	0.198	0.78 (0.55-1.11)	0.164
Chronic respiratory disease	1.15 (0.95-1.38)	0.156	1.16 (0.94-1.45)	0.167
HT without complications	0.85 (0.68-1.06)	0.152	0.68 (0.53-0.89)	0.005
HT with complications	1.29 (1.01-1.66)	0.045	1.06 (0.78-1.430)	0.727
Congestive heart failure	0.78 (0.64-0.95)	0.014	1.00 (0.80-1.25)	0.987
Chronic neurological disease	0.78 (0.65-0.95)	0.012	1.44 (1.18-1.76)	< 0.001
Chronic kidney disease	0.94 (0.76-1.15)	0.535	1.55 (1.26-1.88)	< 0.001
ALT	1.00 (1.00-1.00)	0.420	1.00(1.00 - 1.00)	0.892
ALP	1.00 (1.00-1.00)	0.916	1.02 (1.02-1.03)	< 0.001
AST	1.00 (1.00-1.00)	0.003	1.00 (1.00-1.01)	< 0.001
T-Bil	1.06 (0.99-1.14)	0.008	1.21 (1.14-1.28)	< 0.001
Albumin	0.87 (0.76-1.01)	0.071	0.84 (0.71-1.01)	0.057
INR	0.91 (0.77-1.09)	0.312	1.12 (0.99-1.26)	0.071
PT	1.00 (0.98-1.02)	0.814	1.03 (1.02-1.05)	< 0.001
Neutrophil	1.00 (1.00-1.00)	0.858	1.00 (1.00-1.01)	0.008
BUN	1.01 (1.00-1.01)	<0.001	1.01 (1.01-1.02)	< 0.001
Creatinine	1.07 (1.02-1.13)	0.007	1.16 (1.11-1.22)	< 0.001
Interleukin-6	1.00 (1.00-1.00)	<0.001	1.00 (1.00-1.00)	< 0.001
CRP	1.02 (1.01-1.03)	0.001	1.04 (1.03-1.05)	< 0.001
Ferritin	1.00 (1.00-1.00)	0.002	1.00 (1.00-1.00)	0.001
D-Dimer	1.00 (0.98-1.02)	0.721	1.03 (1.01-1.05)	0.004
LDH	1.00 (1.00-1.00)	0.063	1.00 (1.00-1.01)	< 0.001

[1]Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; HT: Hypertension; ALT: Alanine aminotransferases; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; AST: Aspartate aminotransferase; T-Bil: Total bilirubin; INR: International normalized ratio; BUN: Blood urea nitrogen; PT: Prothrombin time; CRP: C-reactive protein; LDH: Lactate Dehydrogenase; HR:Hazard ratio; CI: Confidence interval.

risk than patients with normal levels. Age, gender, ethnicity, race, BMI, and all the preexisting comorbidities were adjusted as confounders. Among these liver chemistries, elevated AST and T-Bil levels were associated with the highest risk of in-hospital mortality. Compared to patients with T-Bil in the normal level, all-cause mortality risk significantly increased 6-fold (95%CI, 2.90-12.41; $P < 0.001$) in patients with an elevated T-Bil level of >2-5 × ULN and increased 7.86-fold (95%CI, 1.88-32.96; $P = 0.005$) in patients with T-Bil > 5 × ULN (Table 5). A stepwise increase in the levels

Table 5 Association of abnormal liver chemistries and mortality in patients with a positive test for severe acute respiratory syndrome coronavirus-2

Parameters	Unadjusted, Cox regression		Adjusted[1], Cox regression		Log-rank test
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	*P* value
ALT, Abnormality type					
Normal,	Reference		Reference		
1-2 ULN	0.83 (0.65-1.07)	0.146	0.95 (0.72-1.25)	0.719	< 0.001
> 2-5 ULN	0.52 (0.40-0.68)	< 0.001	0.68 (0.53-0.92)	0.013	
> 5 ULN	0.84 (0.64-1.11)	0.219	1.31 (0.98-1.79)	0.092	
AST, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.16 (0.94-1.43)	0.169	1.07 (0.84-1.35)	0.584	0.001
> 2-5 ULN	1.48 (1.11-1.98)	0.008	1.49 (1.06-2.10)	0.021	
> 5 ULN	2.13 (1.37-3.32)	0.001	2.19 (1.27-3.76)	0.005	
Bilirubin, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.74 (1.27-2.40)	0.001	1.58 (1.04-2.22)	0.032	< 0.001
> 2-5 ULN	2.49 (1.24-5.02)	0.011	6.00 (2.90-12.41)	< 0.001	
> 5 ULN	2.78 (0.89-8.65)	0.078	7.86 (1.88-32.96)	0.005	
ALP, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.52 (1.20-1.92)	< 0.001	1.42 (1.09-1.86)	0.009	0.001
> 2-5 ULN	2.13 (1.36-3.35)	0.001	1.81 (1.05-3.10)	0.032	
> 5 ULN	1.05 (0.15-7.45)	0.964	1.84 (0.25-13.38)	0.547	

[1]Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ALT: Alanine aminotransferases; AST: Aspartate aminotransferase; T-Bil: Total bilirubin; ALP: Alkaline phosphatase; HR: Hazard ratio; CI: Confidence interval; ULN: Upper limit of normal.

of AST was associated with a significant increased risk of all-cause mortality (HR, 1.49; 95%CI, 1.06-2.10; $P < 0.001$ for > 2-5 × ULN; HR, 2.19; 95%CI, 1.27-3.76; $P = 0.005$ for AST > 5 × ULN). The degree of ALT ranging from > 2-5 × ULN (adjusted HR, 0.68; 95%CI, 0.53-0.92; $P = 0.013$) was associated with a decreased risk of all-cause mortality; however, 1-2 × and > 5 × ULN were not significantly associated with all-cause mortality. Lastly, compared to the patients with ALP in the normal range, all-cause mortality risk significantly increased 1.42-fold (95%CI, 1.09-1.86; $P = 0.009$) in patients with ALP > 1-2 × ULN and increased 1.81-fold (95%CI, 1.05-3.10; $P = 0.032$) in patients with ALP > 2-5 × ULN after adjusting for confounders. Interestingly, the ALP levels > 5 × ULN were not significantly associated with mortality.

Need for vasopressor support: Only two (0.1%) patients in the non-severe group required vasopressor drugs, whereas 585 (43.2%) patients with severe COVID-19 required vasopressors ($P < 0.001$). Multivariable adjusted Cox proportional hazard regression analysis revealed that patients with abnormal AST (HR, 1.00, 95%CI, 1.00-1.00; $P < 0.001$), T-Bil (HR, 1.09, 95%CI, 1.03-1.17; $P = 0.003$), ALP (HR, 1.02, 95%CI, 1.01-1.03; $P < 0.001$), blood urea nitrogen (BUN) (HR, 1.007, 95%CI, 1.00-1.01; $P < 0.001$), and PT (HR, 0.795, 95%CI, 0.72-0.88; $P < 0.001$) were associated with a need for vasopressor drugs. Similarly, a higher state of inflammation was also associated with this outcome, namely higher levels of ferritin (HR, 1.00, 95%CI, 1.00-1.00; $P = 0.035$), D-Dimer (HR, 1.02, 95%CI, 1.00-1.04; $P = 0.04$), and IL-6 (HR, 1.00, 95%CI, 1.00-1.00; $P < 0.001$). However, higher BMI and abnormal ALT were not independently associated

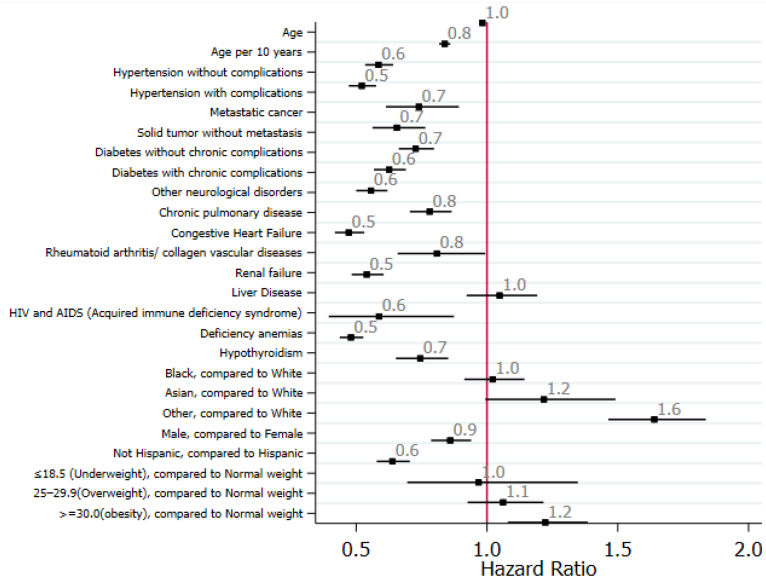


Figure 2 Standardized forest plot comparing selected clinical variables between elevated ALT and normal ALT among hospitalized. ALT: Alanine aminotransferase.

with the increased risk for this outcome. Of note, chronic neurological disease (HR, 0.77, 95%CI, 0.63-0.93; P = 0.004) and albumin (HR, 0.804, 95%CI, 0.69-0.93; P = 0.004) were associated with lower vasopressor support hazards in multivariate analyses.

Need for mechanical ventilation: Patients who received invasive mechanical ventilation were older, more likely to have preexisting comorbidities than were patients who did not receive invasive mechanical ventilation (Table 3). However, preexisting liver diseases did not differ between these groups. Median levels of ALT, AST, and T-Bil were significantly increased for patients who received mechanical ventilation (P < 0.0001) compared to those who did not receive it. However, no difference in ALP and GGT levels were seen between these two groups (ventilated P = 0.091 *vs* non-ventilated patients P = 0.483). Furthermore, patients who received invasive mechanical ventilation had varying degrees of abnormal liver chemistries (1-2 ×, > 2-5 ×, and > 5 × ULN), but in general, ALT, AST, and T-Bil values were significantly higher in these patients (P < 0.001). Multivariable Cox regression analysis adjusted for age, gender, ethnicity, race, BMI, and preexisting comorbidities revealed that older age, HT with complications, and abnormal levels of AST, T-Bil, BUN, and creatinine were associated with mechanical ventilation. In addition, a higher state of inflammation was also associated with this outcome, namely higher levels of neutrophil, ferritin, CRP, and IL-6.

DISCUSSION

This retrospective cohort study is one of the largest and most comprehensive to evaluate liver chemistries and clinical outcomes of hospitalized patients with COVID-19. Overall, the results show that liver injury, assessed by elevated liver enzyme levels, is commonly seen in hospitalized patients with COVID-19 and is associated with the risk of in-hospital mortality and other adverse clinical outcomes, such as the need for vasopressor drugs and mechanical ventilation. The key findings of the study are: (1) There is a high prevalence of liver injury (70.4%), defined by an elevation in ALT levels, in hospitalized patients with COVID-19; (2) Abnormal liver chemistries during hospitalization are strongly associated with mortality (ALT, T-Bil, and ALP); (3) Liver injury measured in patients with COVID-19 on admission is associated with the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil); (4) A strong and independent association of AST, T-Bil, and ALP correlates with the severity of COVID-19 infection; and (5) Elevated inflammatory markers (CRP, IL-6, ferritin, D-dimer, and LDH) are associated with increased risk of disease severity.

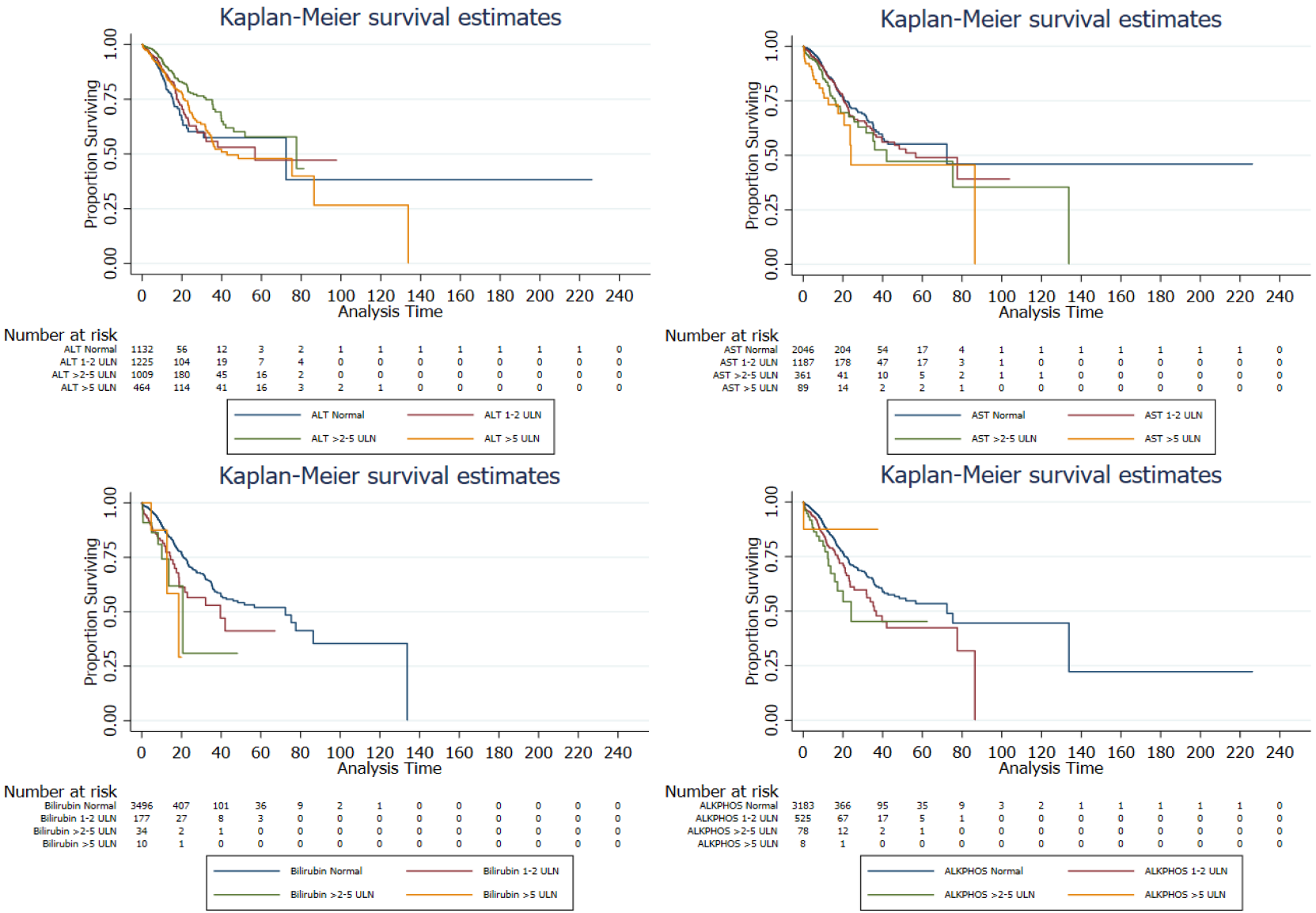


Figure 3 Kaplan-Meier analysis showed the association of abnormal liver chemistry results among patients with COVID-19. ALT: Alanine aminotransferase; AST: aspartate aminotransferase.

The pathophysiology of SARS-CoV-2 infection is similar to other coronavirus infections [SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV)] and shares a large genome sequence homology with other pathogenic human coronaviruses. SARS-CoV and MERS-CoV cause abnormal liver chemistries, and various degrees of hepatic injury[18,19]. SARS-CoV viral RNA is detected in autopsied human liver, suggesting a direct liver involvement[20]. Thus, similar liver injury in COVID-19 patients is not surprising. In particular, the overall ALT elevations were observed in 70.4% of patients with COVID-19 on hospital admission, and 79.3% of patients in the severe group had elevations much higher than previous reports. Based on a recent systematic review and meta-analysis (17 studies, 2711 patients), abnormal ALT is estimated to occur in ~15% of COVID-19 patients[14]. However, in Chinese cohorts, the prevalence of abnormal ALT among patients with COVID-19 ranged between 4% and 53.1%, and recent studies from the United States reveal even higher estimates, ranging between 39% and 64.9%[21-23].

In our cohort, only a small percentage of patients in our study had underlying liver disease (12.2%), suggesting that liver damage in these patients is directly caused by the COVID-19 infection and not an underlying condition or drug-induced liver injury from medications. Furthermore, our study showed varying degrees of liver enzyme levels, ranging from mild to severe. A previous study reported that 9.2% of ALT elevations were > ULN at the time of hospital admission[11], while another study reported higher rates of 36.5% >2 × ULN on admission[24]. Overall, our study showed that 38.8% of patients had abnormal ALT elevations > 2 × ULN (2-5 × ULN 26.7% and > 5 × ULN 12.1%).

Another potential cause of liver injury in our cohort is drug-induced liver injury. Self-medication before hospital admission was not reported in the data and is an unlikely cause. However, during hospitalization, treatment consists of a combination of antibiotics, antivirals, systemic corticosteroids, antipyretics, and analgesics to treat the COVID-19 infection, which may promote liver injury. In this study, these combined drug treatments positively correlated in patients with severe disease. Thus, drug-related liver injury during the treatment of COVID-19 infection needs to be considered. Future studies are needed to evaluate the possible effects of drugs on liver chemistries in patients with COVID-19.

Various studies have shown that older patients with COVID-19 have a higher case fatality rate[25], and having certain comorbidities may contribute to worse outcomes [26]. Similarly, our analysis revealed that risk factors for severe infection and death included older age, chronic heart disease, elevated inflammatory response, more prolonged PT, elevated liver enzymes, and bilirubin. In addition to ALT elevation, AST, T-Bil, and ALP were independently associated with the severity of COVID-19 infection, with over half of severe patients exhibiting AST elevation. The current study is consistent with prior studies that the pattern of liver injury is primarily hepatocellular instead of cholestatic, while our study indicates that elevations in T-Bil and ALP may be more common than previously reported[27]. Upon hospitalization, the percentage of the patients with elevated ALP and T-Bil in severe cases was 18.6% and 6.8%, respectively, which was significantly higher than 14.6 % and 4.6% in patients with the non-severe disease.

COVID-19 causes severe respiratory distress and pneumonia, with the latter being independently correlated with the need for ICU care, mechanical ventilation, and death[28]. Our study complements this knowledge and reveals that elevated liver chemistries can predict the risk of major in-hospital outcomes, such as the need for vasopressor drugs, mechanical ventilation, and death. Moreover, significant hypoalbuminemia was observed, particularly among patients with severe disease, and was also a predictor for the need for vasopressor drugs and mechanical ventilation. In the present study, the elevated levels of LDH, creatinine, BUN, IL-6, CRP, and ferritin are independently associated with mechanical ventilation risk.

COVID-19 induces a release of inflammatory cytokines, leading to organ dysfunction[19]. Inflammatory cytokine storm during COVID-19 infections is not uncommon and can result in sudden patient clinical deterioration and multiorgan failure. Direct hepatocyte injury caused by the SARS-CoV-2 may be closely related to systemic inflammatory response syndrome, and overproduction of cytokines is linked to the lung-liver axis[20]. An increase in systemic immune mediators that cause inflammation, oxidative stress, and underlying hypoxia facilitates and exacerbates liver function[29]. Our findings indicate an association between elevated inflammatory markers (CRP, IL-6, ferritin, D-dimer, and LDH) with increased risk of disease severity. IL-6 values are increased in patients with both severe COVID-19 and significantly increased in patients with elevated liver chemistries compared to patients with non-severe COVID-19. IL-6 is the primary driver of cytokine release syndrome,

and IL-6 inhibitors are effective in treating severe COVID-19 cases[30]. Moreover, the neutrophil levels and serum CRP are significantly increased in patients with liver injury from COVID-19. These data imply a potential association between liver injury and the inflammatory responses induced by SARS-CoV-2 infection. Clinical treatment against the cytokine storm might also reduce liver injury and liver injury-related mortality. We also found a reduction in red blood cells in severe patients, which coincides with the fact that SARS-CoV-2 destroys hemoglobin in red blood cells, dissociates deoxyhemoglobin and iron, and produces hypoxia and respiratory distress. Increased ferritin levels due to cytokine storm and secondary hemophagocytic lymphohistiocytosis have also been reported in severe COVID-19 patients[31]. A higher level of ferritin was observed in patients with severe disease on admission than patients with a non-severe disease in the present study. Hypoxia may also damage hepatocytes and induce liver injury; thus, elevated levels of serum ferritin and hypoxia are potential indicators of hepatocyte injury in patients with severe COVID-19 infection. In our study, abnormal levels of LDH were found in patients with severe COVID-19, which was also seen in patients with SARS and MERS and was an independent risk factor for severe disease[32]. LDH is an intracellular enzyme found in cells in almost all organ systems and can be released during tissue damage, and is involved in various pathophysiological processes. Abnormal levels of LDH seem to reflect that multiple organ injury and failure. Despite its lack of specificity, serum LDH can have great prognostic significance in patients with COVID-19.

Limitations: Despite analyzing a large cohort of patients, the study has some limitations. Data collection was a retrospective observational cohort study and used electronic health record extraction within a single health system. Our health system is a tertiary medical health system, potentially introducing referral bias. The analysis represents only patients who were hospitalized, *i.e.*, more likely to be in severe cases. Therefore, it cannot be entirely excluded that abnormal liver chemistries at admission might represent a more severe course in patients with COVID-19 with multiorgan involvement, including hepatobiliary manifestations. However, irrespective of the cause of liver injury at the time of hospitalization, we show a strong association between severity and liver chemistries at hospital admission rather than peak values, which may help guide clinical decisions early in the disease course. Furthermore, it was not feasible to describe all the potential causes of liver injury and all the causes of liver injury in the patients progressing to liver injury, such as the use of hepatotoxic medications and self-medication before hospitalization. Our study's data permit an initial evaluation of patients' clinical course and outcomes with COVID-19. The causes of death in COVID-19 patients may involve multiple organ injuries, and it is challenging to differentiate liver injury as the primary and direct cause of death. We were unable to obtain long-term outcomes due to a comparatively short observation period. Further studies with long-term periods are required to understand the long-term impact of COVID-19 on the liver and elucidate the pathogenic mechanisms.

CONCLUSION

This study found that abnormal liver chemistries (AST, ALT, T-Bil, and ALP) at the time of hospital admission are associated with worse outcomes in COVID-19 patients, namely mortality (ALT, T-Bil, and ALP), the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil). Consequently, in hospitalized COVID-19 patients, elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies.

ARTICLE HIGHLIGHTS

Research background

Severe acute respiratory syndrome coronavirus 2 primarily infects the respiratory system. Abnormal liver chemistries are common findings in patients with Coronavirus Disease 2019 (COVID-19). In addition, increasing evidence exists for the direct multiorgan effect. However, the association of these abnormalities with the severity of COVID-19 and clinical outcomes is poorly understood.

Research motivation

To explore the impact of abnormal liver chemistries in hospitalized patients with COVID-19 and whether it is associated with worse outcomes, namely mortality, the need for vasopressor drugs, and mechanical ventilation.

Research objectives

We examine whether abnormal liver chemistries in COVID-19 hospitalized patients can be of prognostic value. We determined the prevalence of elevated liver chemistries in a large cohort of hospitalized patients with COVID-19 infection and identified whether an independent association exists between abnormal liver chemistries and clinical severity or the risk of in-hospital mortality.

Research methods

This retrospective, observational study included 3380 patients with COVID-19 who were hospitalized in the Johns Hopkins Health System. Demographic data, clinical characteristics, laboratory findings, treatment measures, and outcome data were collected. Cox regression modeling was used to explore variables associated with abnormal liver chemistries on admission with disease severity and prognosis.

Research results

A total of 2698 (70.4%) had abnormal ALT at the time of admission. Other more prevalent abnormal liver chemistries were AST (44.4%), ALP (16.1%), and T-Bil (5.9%). Factors associated with liver injury were older age, Asian ethnicity, other race, being overweight, and obesity. Higher ALT, AST, T-Bil, and ALP levels were more commonly associated with disease severity. Multivariable adjusted Cox regression analysis revealed that abnormal AST and T-Bil were associated with the highest mortality risk than other liver injury indicators during hospitalization. Abnormal AST, T-Bil and ALP were associated with a need for vasopressor drugs whereas, higher levels of AST, T-Bil, and a decreased albumin levels were associated with mechanical ventilation.

Research conclusions

This study found that abnormal liver chemistries (AST, ALT, T-Bil, ALP, and albumin) at the time of hospital admission are associated with worse outcomes in COVID-19 patients, namely mortality (ALT, T-Bil, and ALP), the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil). Consequently, in hospitalized COVID-19 patients, elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies.

Research perspectives

Abnormal liver chemistries are common at the time of hospital admission are associated with worse outcomes in COVID-19 patients. In particular, abnormal levels of AST, T-Bil, ALP, and hypoalbuminemia correlate with the severity of COVID-19 infection, and abnormal liver chemistries (ALT, T-Bil, and ALP) during hospitalization are strongly associated with all-cause mortality in patients with COVID-19. Furthermore, liver injury measured in patients with COVID-19 on admission is associated with the need for vasopressor drugs (AST, T-Bil, and ALP) and mechanical ventilation (AST, and T-Bil).

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CASE REPORT

Simultaneous endoscopic and video-assisted retroperitoneal debridement in walled-off pancreatic necrosis using a laparoscopic access platform: Two case reports

Lars Lindgaard, Morten Laksáfoss Lauritsen, Srdan Novovic, Erik Feldager Hansen, John Gásdal Karstensen, Palle Nordblad Schmidt

ORCID number: Lars Lindgaard 0000-0001-7275-3093; Morten Laksáfoss Lauritsen 0000-0002-5824-8207; Srdan Novovic 0000-0001-6246-874X; Erik Feldager Hansen 0000-0003-4323-4133; John Gásdal Karstensen 0000-0001-9333-0399; Palle Nordblad Schmidt 0000-0001-9243-8824.

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Lars Lindgaard, Morten Laksáfoss Lauritsen, Srdan Novovic, Erik Feldager Hansen, John Gásdal Karstensen, Palle Nordblad Schmidt, Pancreatitis Centre East (PACE), Gastro Unit,Copenhagen University Hospital–Amager and Hvidovre, Hvidovre 2650, Capital Region, Denmark

Morten Laksáfoss Lauritsen, Srdan Novovic, John Gásdal Karstensen, Department of Clinical Medicine, University of Copenhagen, Copenhagen 2100, Capital Region, Denmark

Corresponding author: John Gásdal Karstensen, MD, PhD, Associate Professor, Chief Physician, Surgeon, Pancreatitis Centre East (PACE), Gastro Unit, Copenhagen University Hospital–Amager and Hvidovre, Kettegård Alle 30, Hvidovre 2650, Capital Region, Denmark. john.gasdal.karstensen@regionh.dk

Abstract

BACKGROUND

Infected walled-off necrosis is a potentially life-threatening complication of necrotizing pancreatitis. While some patients can be treated by drainage alone, many patients also need evacuation of the infected debris. Central necroses in relation to the pancreatic bed are easily reached *via* an endoscopic transluminal approach, whereas necroses that involve the paracolic gutters and the pelvis are most efficiently treated *via* a percutaneous approach. Large and complex necroses may need a combination of the two methods.

CASE SUMMARY

Transluminal and percutaneous drainage followed by simultaneous endoscopic and modified video-assisted retroperitoneal debridement was carried out in two patients with very large (32-38 cm), infected walled-off necroses using a laparoscopic access platform. After 34 d and 86 d and a total of 9 and 14 procedures, respectively, complete regression of the walled-off necroses was achieved. The laparoscopic access platform improved both access to the cavities as well as the overview. Simultaneous transluminal and percutaneous necrosectomy are feasible with the laparoscopic access platform serving as a useful adjunctive.

CONCLUSION

This approach may be necessary to control infection and achieve regression in some patients with complex collections.

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Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Core Tip: Infected walled-off necrosis is a potentially life-threatening complication of necrotizing pancreatitis. In cases with large or complex walled-off necrosis, treatment combining endoscopic, transluminal and percutaneous video-assisted retroperitoneal debridement may be needed. We report on two patients who underwent a combination of endoscopic and percutaneous drainage and necrosectomy for their large, infected walled-off necroses. Furthermore, we introduce a laparoscopic access platform as a useful adjunctive to endoscopic necrosectomy and video-assisted retroperitoneal debridement.

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INTRODUCTION

Infected walled-off necrosis (WON) is a potentially life-threatening complication of necrotizing pancreatitis. Traditionally, such patients were treated *via* open surgery, but during the last decade an endoscopic or surgical step-up approach using minimally invasive techniques has become the treatment of choice[1-5].

Whereas some patients can be treated by drainage alone, many patients also need evacuation of the infected debris. In such cases, it is our experience that central necroses in relation to the pancreatic bed are most easily reached by endoscopic, transluminal drainage followed by endoscopic necrosectomy (EN), whereas necroses that involve the paracolic gutters and the pelvis are more effectively treated with percutaneous drainage and video-assisted retroperitoneal debridement (VARD).

We report on two patients who needed a combination of endoscopic and percutaneous drainage and necrosectomy for their large, infected WONs. Furthermore, we introduce a laparoscopic access platform (Figures 1 and 2) as a useful adjunctive to EN and VARD.

CASE PRESENTATION

Chief complaints

Case 1: A 36-year-old male with a history of proctocolectomy and J-pouch due to ulcerative colitis was admitted to a local hospital because of jaundice.

Case 2: An 18-year-old boy was admitted to another hospital with severe necrotizing pancreatitis of unknown etiology.

History of present illness

Case 1: Magnetic resonance imaging raised suspicion of a cholangiocarcinoma in the liver hilum, a diagnosis that was subsequently confirmed. An endoscopic retrograde cholangiopancreatography revealed multiple intra- and extrahepatic bile duct strictures consistent with primary sclerosing cholangitis but failed to relieve the obstruction. Subsequent percutaneous transhepatic biliary drainage was carried out. After the endoscopic retrograde cholangiopancreatography, the patient developed post-endoscopic retrograde cholangiopancreatography pancreatitis, severe sepsis, and multiple organ failure and was admitted to the intensive care unit (ICU).



Figure 1 GelPOINT path transanal access platform.

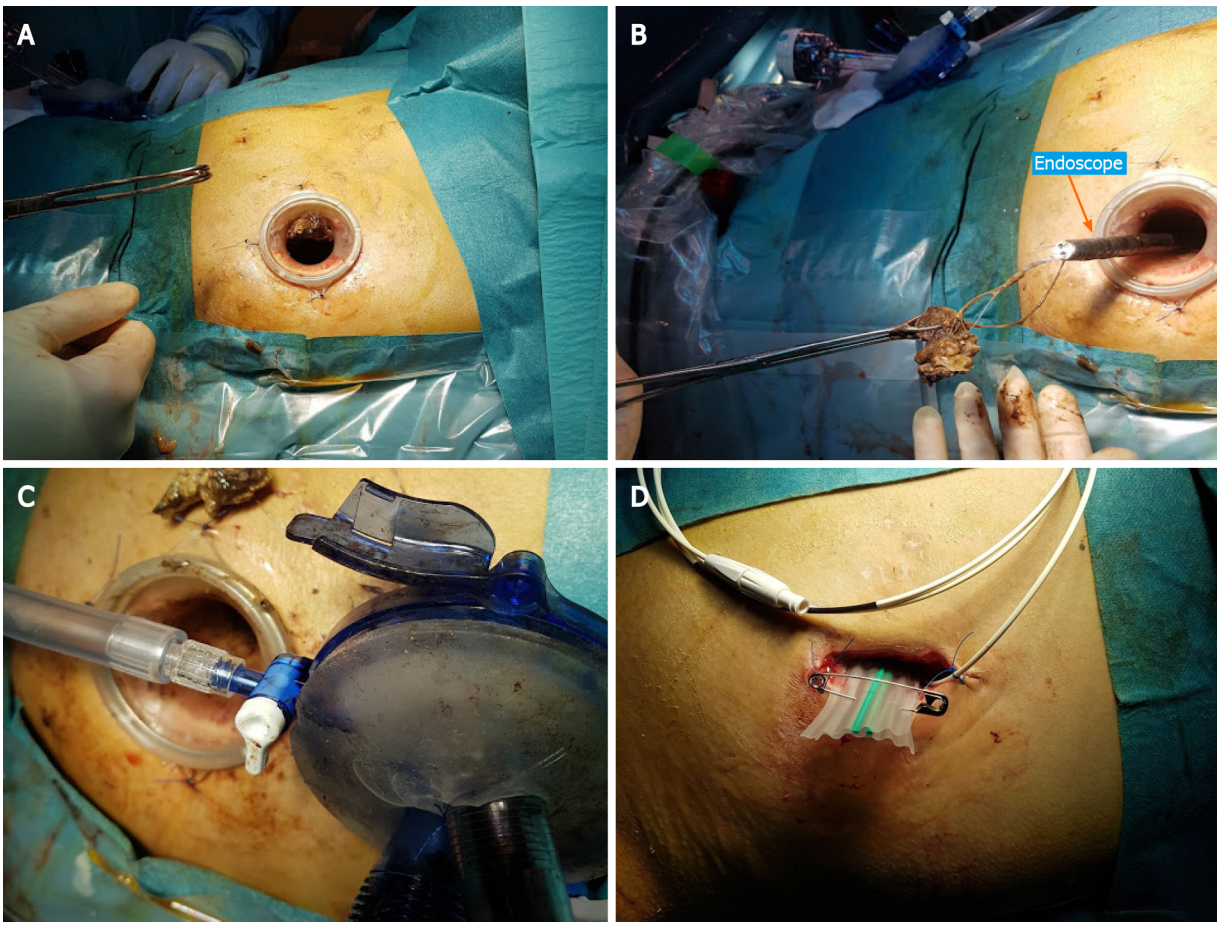


Figure 2 Endoscopic-laparoscopic retroperitoneal debridement. A: GelPOINT path transanal access platform with access channel placed in the video-assisted retroperitoneal debridement incision; B and C: Necrotic debris evacuated through the access channel during endoscopic, transluminal necrosectomy; D: Corrugated drainage sheet placed in the video-assisted retroperitoneal debridement incision at the end of the procedure.

Case 2: On day 2, he was referred to the ICU with respiratory and circulatory failure. On day 3, he developed intra-abdominal hypertension and renal failure with the need for dialysis.

FINAL DIAGNOSIS

Case 1: After 6 wk, which included severe episodes of gastrointestinal bleeding that needed coiling and laparotomy with creation of an ileostomy, he was referred to our hospital for endoscopic drainage of a large (12 cm × 14 cm × 38 cm), infected, left-sided WON extending from the spleen down through the left paracolic gutter into the left groin (Figure 3A).

Case 2: On day 43, he was referred to the ICU at our hospital with septicemia and a large (16 cm × 24 cm × 32 cm), infected, left-sided WON extending from the pancreatic bed into the pelvis (Figure 4A).

TREATMENT

Case 1: Between days 57 and 143, the patient underwent one endoscopic transluminal drainage, four EN, two VARD, and seven combined EN/VARD procedures.

Case 2: The same day, VARD was performed. Five days later, after only one VARD and one EN session, he was discharged from the ICU. The patient underwent a total of one endoscopic transluminal drainage, four VARD, two EN and two combined VARD/EN procedures, with the last procedure occurring on day 77.

OUTCOME AND FOLLOW-UP

Case 1: There was complete regression of the large WON (Figure 3B).

Case 2: There was complete regression of the large WON (Figure 4B).

DISCUSSION

In this paper, we describe 2 patients with necrotizing pancreatitis who needed treatment with a combination of EN and VARD. Both patients were referred to our hospital with signs of sepsis due to very large, infected WONs. We chose to combine two minimally invasive techniques to control the infection as quickly as possible in order to accelerate the treatment course.

EN is ideal for treating central WONs, whereas VARD is especially suitable in the case of lateral collections extending into the paracolic gutters and the pelvis. Even though flexible endoscopes can reach remote parts of the retroperitoneum, an isolated endoscopic approach can prove more time-consuming and delay evacuation of large collections as compared to a combined approach. Furthermore, large fluid collections may become compartmentalized over the course of treatment, creating isolated portions of the WON that can no longer be reached *via* the transluminal route. A successful combination of EN and VARD has been described elsewhere[6,7].

VARD has been thoroughly described in the literature[8,9]. However, use of a laparoscopic platform has not to our knowledge been documented. With the GelSeal cap closed and CO_2 insufflation through the platform, the large cavity could be expanded during the procedure, thus making debridement easier. During transgastric necrosectomy, debris was removed through the access channel of the platform by the surgeon or endoscopy assistants, reducing the procedure time. Finally, the platform facilitated debridement *via* the percutaneous route using the flexible endoscope. The success of the method needs to be investigated in prospective studies evaluating the risk of recurrence and fistula formation as well as matters relating to costs of the technique.

CONCLUSION

We conclude that simultaneous transluminal and percutaneous necrosectomy is practical and hypothesize that it may accelerate treatment in cases of complex WONs due to acute pancreatitis. Furthermore, a laparoscopic access platform is a useful adjunctive to the procedure.

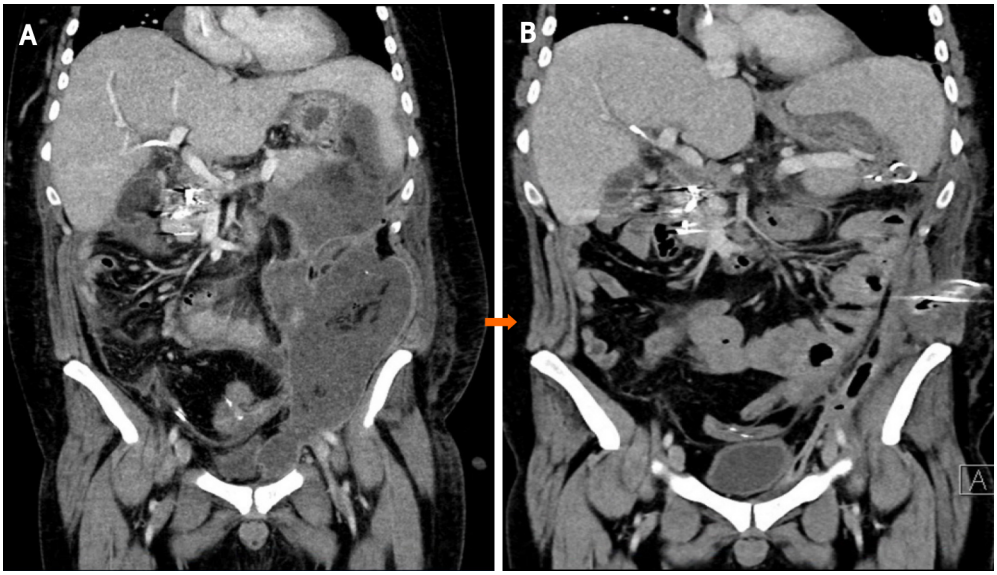


Figure 3 Case 1. Coronal computed tomography with walled-off necrosis. A: Before drainage; B: After 86 d and 14 procedures.

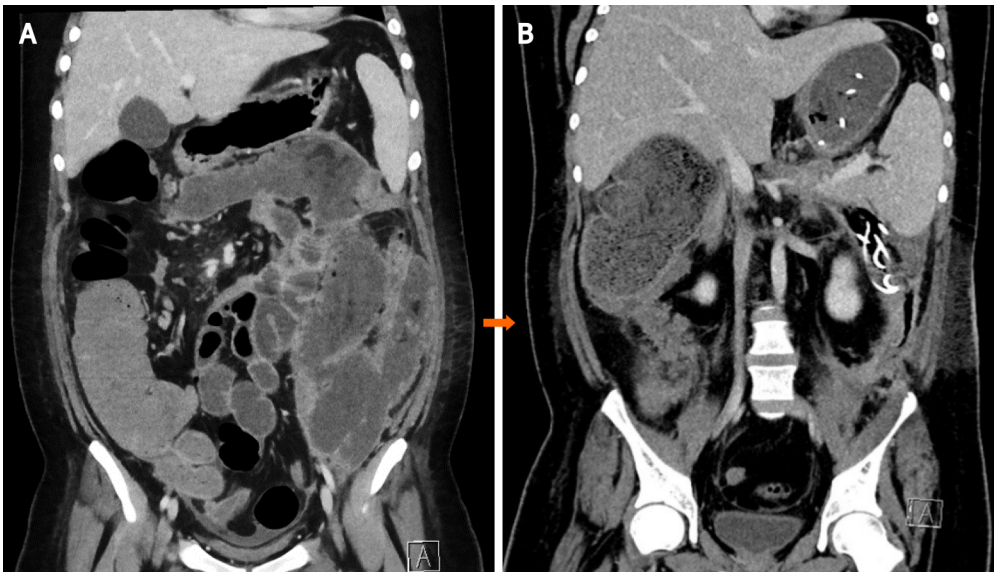


Figure 4 Case 2. Coronal computed tomography with walled-off necrosis. A: Before drainage; B: After 34 d and nine procedures.

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CASE REPORT

Curative resection with endoscopic submucosal dissection of early gastric cancer in *Helicobacter pylori*-negative Ménétrier's disease: A case report

Koh Fukushi, Kenichi Goda, Hitoshi Kino, Masayuki Kondo, Mimari Kanazawa, Ken Kashima, Akira Kanamori, Keiichiro Abe, Tsunehiro Suzuki, Keiichi Tominaga, Hidetsugu Yamagishi, Atsushi Irisawa

ORCID number: Koh Fukushi 0000-0001-8079-3386; Kenichi Goda 0000-0002-0350-3151; Hitoshi Kino 0000-0002-7304-5635; Masayuki Kondo 0000-0002-4567-1444; Mimari Kanazawa 0000-0003-2243-4267; Ken Kashima 0000-0001-6425-4876; Akira Kanamori 0000-0001-9698-9957; Keiichiro Abe 0000-0002-6087-8697; Tsunehiro Suzuki 0000-0002-8033-6414; Keiichi Tominaga 0000-0002-3716-3386; Hidetsugu Yamagishi 0000-0002-6663-8723; Atsushi Irisawa 0000-0002-2271-2717.

Author contributions: Fukushi K reviewed the literature and contributed to manuscript writing and data analysis; Goda K and Irisawa A contributed to draft conception and design; Fukushi K, Kino H, Kondo M, Kanazawa M, Kashima K, Kanamori A, Abe K, Suzuki T, Tominaga K, and Goda K were the clinicians involved in patient diagnostics, management, therapy, and surveillance; Yamagishi H performed pathological diagnosis; all authors have read and approved the final manuscript.

Informed consent statement: A written informed consent was obtained from the patient at the time of ESD.

Koh Fukushi, Kenichi Goda, Hitoshi Kino, Masayuki Kondo, Mimari Kanazawa, Ken Kashima, Akira Kanamori, Keiichiro Abe, Tsunehiro Suzuki, Keiichi Tominaga, Atsushi Irisawa, Department of Gastroenterology, Dokkyo Medical University, Mibu 321-0293, Tochigi, Japan

Hidetsugu Yamagishi, Department of Diagnostic Pathology, Dokkyo Medical University, Mibu 321-0293, Tochigi, Japan

Corresponding author: Kenichi Goda, MD, PhD, Professor, Department of Gastroenterology, Dokkyo Medical University, 880 Kitakobayashi, Mibu 321-0293, Tochigi, Japan. goda@dokkyomed.ac.jp

Abstract

BACKGROUND

Adult-onset Ménétrier's disease is strongly associated with *Helicobacter pylori* (*H. pylori*) infection and an elevated risk of carcinogenesis. Cases of early-stage gastric cancer developed in *H. pylori*-negative Ménétrier's disease are extremely rare. We report a case of early gastric cancer in *H. pylori*-negative Ménétrier's disease that was curatively resected with endoscopic submucosal dissection (ESD).

CASE SUMMARY

A 60-year-old woman was referred to our hospital after her medical examination detected anemia. Contrast-enhanced upper gastrointestinal (UGI) radiography revealed translucency of the nodule-aggregating surface with giant rugae. Blood tests showed hypoproteinemia and were negative for serum *H. pylori* immunoglobulin G antibodies. The ^{99m}Tc-DTPA-human serum albumin scintigraphy showed protein loss from the stomach. UGI endoscopy showed a 40-mm protruding erythematous lesion on giant rugae of the greater curvature of lower gastric body, suggesting early-stage gastric cancer due to Ménétrier's disease. *En bloc* resection with ESD was performed for diagnosis and treatment. Histology of ESD showed well-differentiated tubular adenocarcinoma. The cancer was confined to the mucosa, and complete curative resection was achieved. Foveolar hyperplasia and atrophy of the gastric glands were observed in non-tumor areas, histologically corresponding to Ménétrier's disease. Three years after ESD, gastric cancer had not recurred, and Ménétrier's disease remained in remission with spontaneous regression of giant gastric rugae.

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CONCLUSION
Complete curative resection was achieved through ESD in a patient with early-stage gastric cancer and *H. pylori*-negative Ménétrier's disease.

Key Words: Ménétrier's disease; *Helicobacter pylori*; Gastric cancer; Endoscopic resection; Endoscopic submucosal dissection; Case report

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Core Tip: We reported an extremely rare case of gastric cancer as a complication of *Helicobacter pylori*-negative Ménétrier's disease. The cancer was early-stage, and a minimally invasive curative resection was achieved with endoscopic submucosal dissection (ESD). There was no recurrence, and the patient's course was extremely good. This case also highlights that when endoscopic biopsy tissue from a cancer-suspected lesion showed atypical glands in Ménétrier's disease, total biopsy with ESD can be useful for diagnosis as well as treatment. ESD seems to be a beneficial therapy for early-stage gastric cancer in Ménétrier's disease because of its low invasiveness and high complete curative resection rate.

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INTRODUCTION

Ménétrier's disease (discovered in 1888) is a relatively rare gastric disease[1] characterized by giant rugae in the stomach accompanied by hypoproteinemia. Adult-onset Ménétrier's disease is strongly associated with *Helicobacter pylori* (*H. pylori*) infection and an elevated risk of carcinogenesis[2]. Our literature review suggests that *H. pylori*-negative cases of early-stage gastric cancer are extremely rare[3,4].

We report the first case of *H. pylori*-negative Ménétrier's disease with early gastric cancer in which complete curative resection was achieved through endoscopic submucosal dissection (ESD).

CASE PRESENTATION

Chief complaints

A 60-year-old woman was referred to our hospital for examination and treatment of anemia.

History of present illness

After observing transient melena, the patient's previous doctor conducted a medical examination and detected severe anemia (blood hemoglobin 6.1 g/dL).

History of past illness

The patient had undergone successful treatment of chronic hepatitis C with interferon therapy performed by a previous doctor.

Personal and family history

The patient had no history of smoking or drinking alcohol. There was no relevant family history.

Physical examination

On admission, the patient's temperature was 36.7 °C, heart rate was 81 bpm, blood pressure was 128/80 mmHg. There was conjunctival pallor and no spontaneous abdominal pain or no tenderness.

Laboratory examinations

The blood test revealed hypoproteinemia (serum albumin, 3.4 g/dL), and the serum *H. pylori* immunoglobulin G antibody level was negative (< 3).

Imaging examinations

Computed tomography (CT) of the abdomen revealed marked mucosal thickening of the body of the stomach, with no enlarged lymph nodes around the stomach (Figure 1A). Upper gastrointestinal (UGI) radiograph showed giant gastric rugae[5] and translucency of the nodule-aggregating surface in the greater curvature of the lower gastric body (Figure 1B). The ^{99m}Tc-DTPA-human serum albumin (HSA-D) scintigraphy showed protein loss from the stomach after 24 h (Figure 1C).

Endoscopic examination

UGI endoscopy revealed giant rugae in the gastric body, similar to contrast-enhanced radiographic findings (Figure 2A). A 40-mm broad-based, protruding erythematous lesion with lobular surface was observed on the giant rugae of the gastric body (Figure 2B). UGI endoscopy showed no evidence of atrophic gastritis and intestinal metaplasia.

Endoscopic ultrasonography (EUS) demonstrated the five layers of the gastric wall. In the lesion area, hypertrophy was observed in the first two layers (equivalent to the mucosa and the muscularis mucosae); however, no noticeable structural changes were evident in the third layer (equivalent to the submucosal layer) or deeper (Figure 2C). These findings were consistent with early tumor confined to the mucosal layer.

Low-magnification narrow-band imaging (NBI) showed granular surfaces with various sizes/forms and dilated vessels (Figure 2D). High-magnification NBI demonstrated irregular microstructures of various forms and tortuous microvessels with changes in caliber (Figure 2E).

Histopathological findings

Histological diagnosis based on endoscopic biopsy was Group 2 (non-tumorous changes suspected, though tumorous lesions cannot be ruled out)[6]. Considering the tumor size and high probability of a cancerous lesion based on the endoscopic images, *en bloc* resection with ESD was performed for definitive diagnosis and treatment (Figure 3A). Loupe image showed that the well-differentiated tubular adenocarcinoma was confined to the mucosal layer, with no lymphovascular invasion (ly0/v0) or ulceration (UL0), and both the lateral and vertical margins were negative (Figure 3B).

Histology of ESD specimens also showed proliferation of atypical cells with an irregular glandular structure (Figure 4A and B). Additionally, scattered p53 protein-positive cells were observed (Figure 4C). Based on these findings, the atypical glands were diagnosed with early gastric cancer of a well-differentiated tubular adenocarcinoma. Foveolar hyperplasia and atrophy of the proper gastric glands were observed in the non-tumor areas, a histological image corresponding to Ménétrier's disease (Figure 4D).

Immunohistochemical staining showed that the tumor cells were diffusely positive for MUC5AC (Figure 5A) and partially positive for MUC6 (Figure 5B), and negative for MUC2 (Figure 5C) and CD10 (Figure 5D). *H. pylori* were not observed in any biopsy or ESD specimens.

FINAL DIAGNOSIS

The final diagnosis of the presented case was early-stage gastric cancer and *H. pylori*-negative Ménétrier's disease.

TREATMENT

The patient underwent ESD.

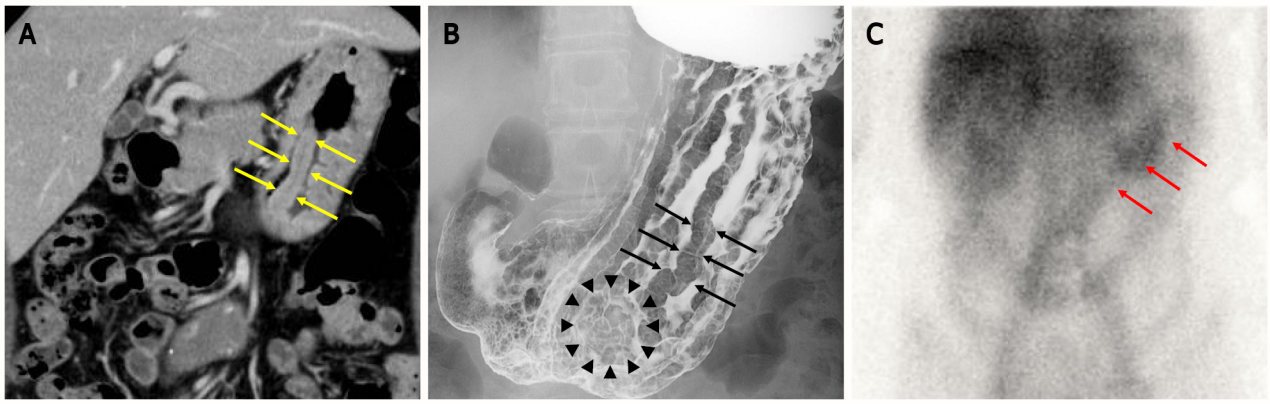


Figure 1 Images of computed tomography scan, upper-gastrointestinal radiograph and scintigraphy. A: Computed tomography scan revealed marked mucosal thickening of the body of the stomach (yellow arrow); B: The double-contrast technique shows remarkably enlarged fold width > 10 mm (black arrow) and translucency of nodule aggregating surface at the greater curvature in the lower gastric body (black arrowhead); C: ^{99m}Tc-DTPA-human serum albumin (HSA-D) scintigraphy: The arrows indicate accumulation in the stomach (red arrow).

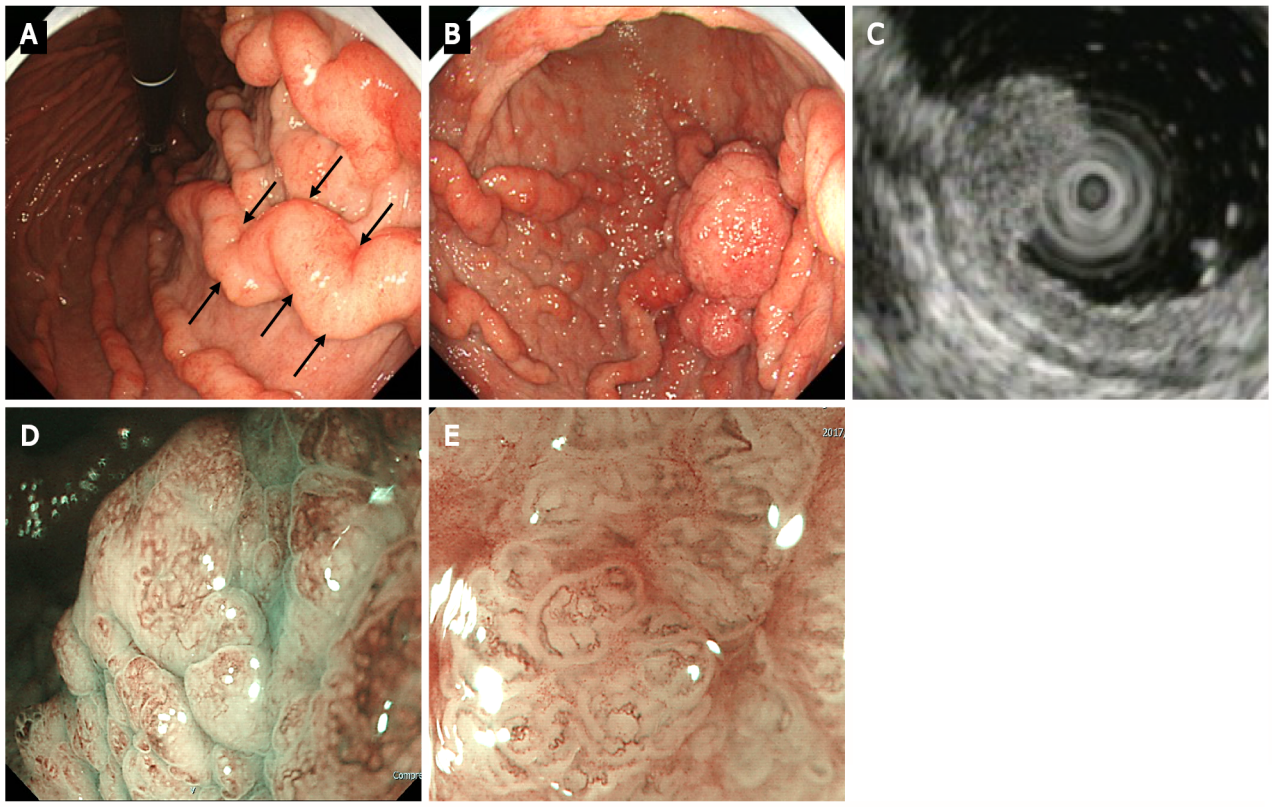


Figure 2 Upper gastrointestinal endoscopy. A: Upper gastrointestinal endoscopy shows marked fold enlargement from the ventricular angle to the fundus (black arrow); B: A 40-mm broad-based protruding lesion is observed near the posterior wall of the greater curvature of the lower gastric body; C: Endoscopic ultrasonography shows the five layers of the gastric wall. In the lesion area, hypertrophy was observed in the first two layers. No noticeable structural changes were evident in the third layer or deeper; D: Low-magnification narrow-band imaging (NBI) shows granular surfaces with various sizes/forms and dilated vessels; E: High-magnification NBI demonstrates irregular microstructures with various forms and tortuous microvessels with changes in caliber.

OUTCOME AND FOLLOW-UP

Histology of ESD specimens showed complete curative resection. Endoscopic follow-up was performed twice a year for 3 years after ESD, and no recurrence was detected (Figure 6). Even though no therapeutic agents were administered specifically for Ménétrier's disease, the giant rugae regressed spontaneously, hypoproteinemia and anemia improved gradually, and the remission was maintained until the last surveillance endoscopy.

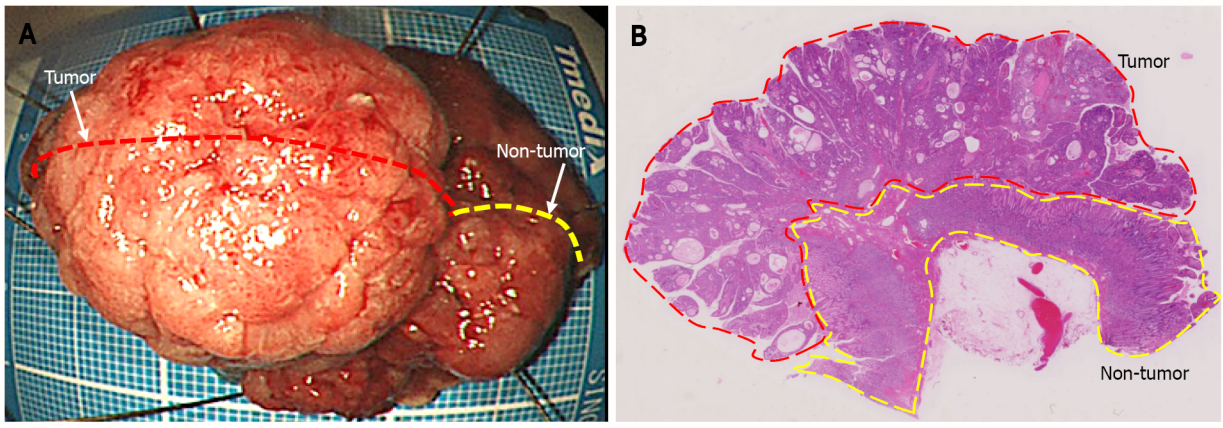


Figure 3 Endoscopic submucosal dissection specimen. A: 45 mm × 38 mm resection specimen comprising a broad-based protruding lesion with nodular surface and uninvolved (non-tumor) tissue with giant rugae; B: Loupe image shows that the well-differentiated tubular adenocarcinoma was confined to the mucosal layer, with no lymphovascular invasion (ly0/v0) or ulceration (UL0), and both the lateral and vertical margins were negative.

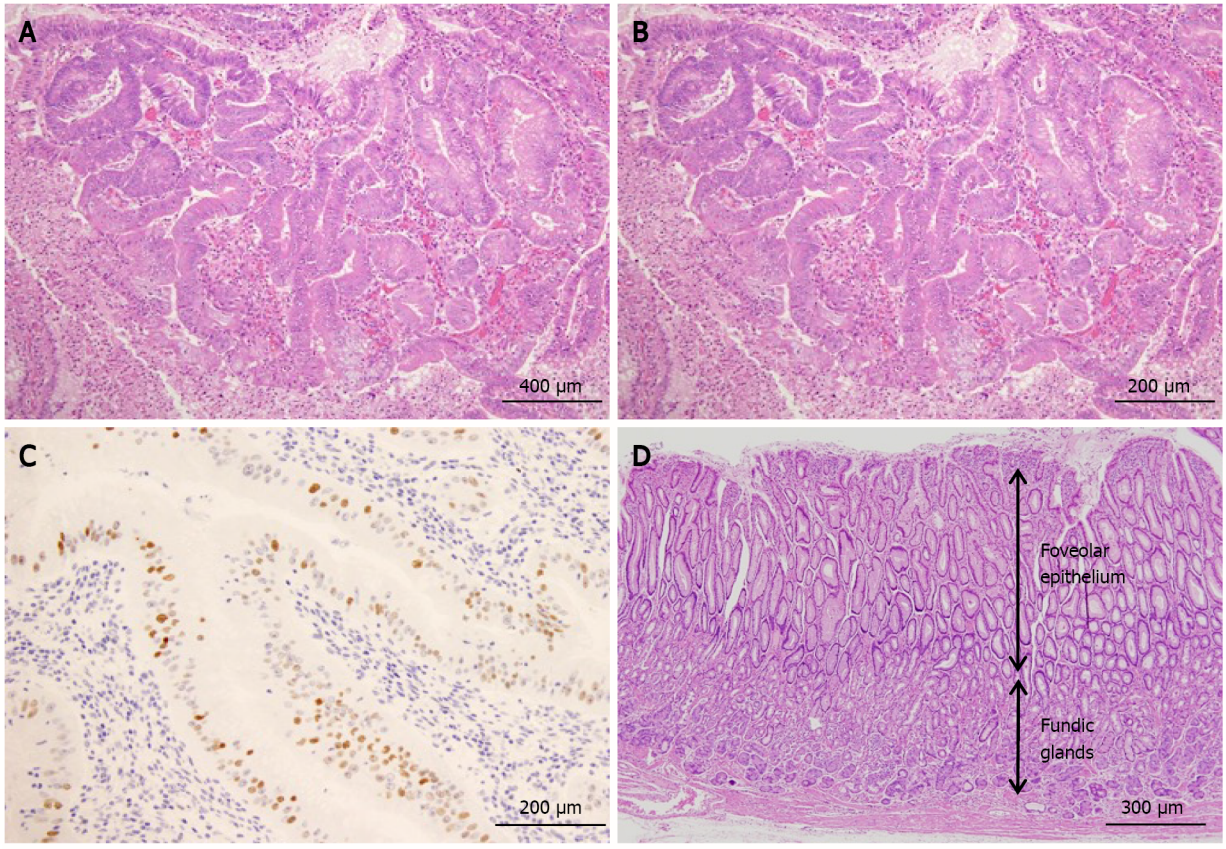


Figure 4 Histological findings. A: The protruding lesion exhibits proliferation of atypical glands with irregular tubular structures (HE; × 20); B: High magnification shows proliferation of atypical cells and enlarged nuclei (irregular or oval-shaped) in the irregular tubular structure (HE; × 20); C: Scattered positive image (in nuclei) in the region (p53; × 20); D: Foveolar hyperplasia and atrophy of the proper gastric glands in non-cancerous area, consistent with Ménétrier's disease (HE; × 20).

DISCUSSION

We reported an extremely rare case of early gastric cancer as a complication of *H. pylori*-negative Ménétrier's disease. We succeeded in curative resection of the cancerous lesion with ESD. Three years after the ESD, there was no recurrence of gastric cancer and Ménétrier's disease regressed spontaneously and had maintained regression for about 30 mo.

Although the present case was *H. pylori*-negative, there have been two reports of close associations between adult-onset Ménétrier's disease and *H. pylori* infection[7,8].

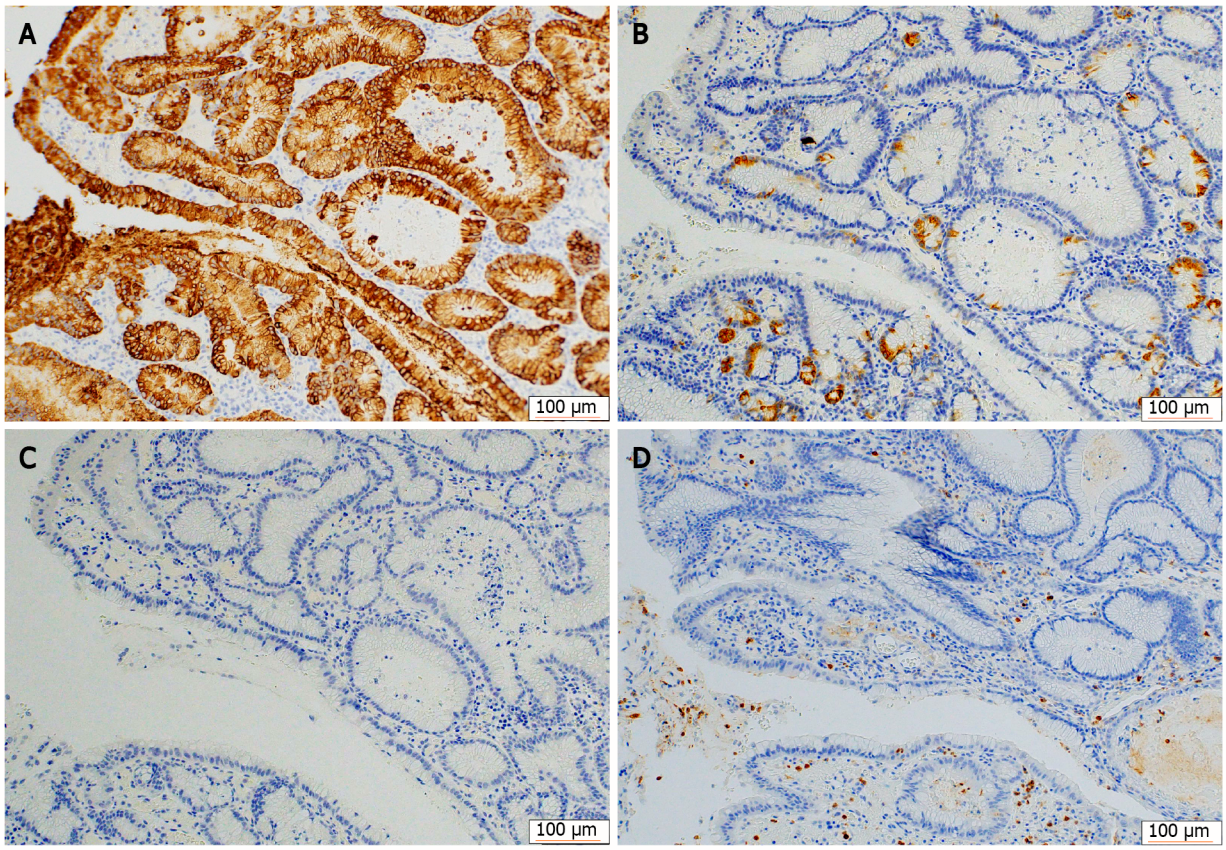


Figure 5 Immunohistochemical staining for mucin phenotypes. The tumor cells were diffusely positive for MUC5AC and partially positive for MUC6, and negative for MUC2 and CD10. A: MUC5AC; B: MUC6; C: MUC2; D: CD10.

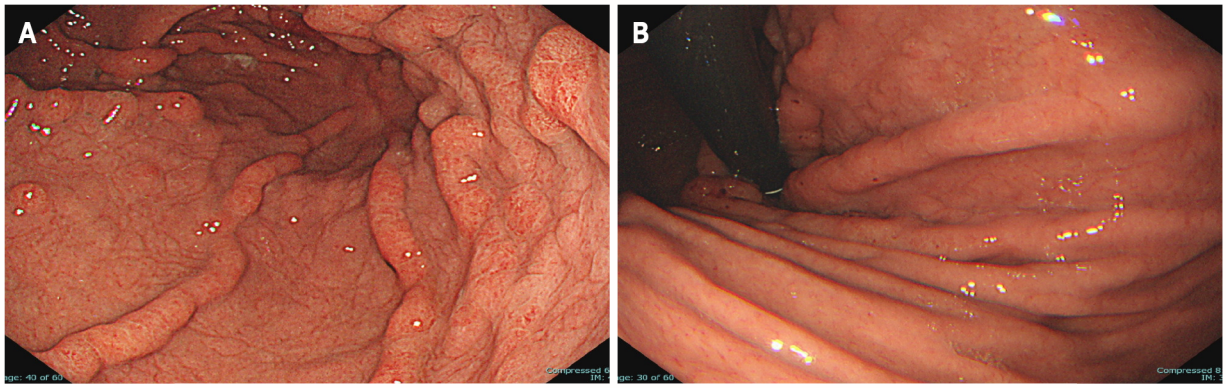


Figure 6 Uppeer-gastrointestinal endoscopy 3 yr after endoscopic submucosal dissection. A: Endoscopic submucosal dissection scar but no recurrence of tumor; B: The giant rugae with erythematous and edema has regressed spontaneously. Ménétrier's disease has remained in remission for the past 3 yr.

It has been reported that Ménétrier's disease with hypertrophic gastropathy increases the risk of gastric carcinogenesis[9]; however, the association between gastric carcinogenesis and *H. pylori* infection in Ménétrier's disease remains unclear.

There have been about 80 case reports on gastric cancer (including advanced cancer) in adult-onset Ménétrier's disease, though few have reported early-stage cancers. Our literature search found four case reports of Ménétrier's disease with early gastric cancer and recorded endoscopic findings and *H. pylori* infection status (Table 1); two of these cases were *H. pylori*-positive[10,11], and three cases, including the present one, were *H. pylori*-negative[3,4]. Our case was the second early gastric cancer to undergo endoscopic resection and the first resection with ESD instead of conventional endoscopic mucosal resection. Macroscopically, all the lesions of early gastric cancer were of protruding-type[12] and located on the oral side of the ventricular angle (corpus/fundus region)[13]. Hypoalbuminemia was observed in three cases and was

Table 1 Case reports on early-stage gastric cancer in patients with Ménétrier's disease

Ref.	Age	Gender	Hypoalbuminemia[1] (g/L)	*H. pylori*	Differentiation	Tumor invasion depth	Macroscopic types	Tumor location	Therapy	Outcome
Raderer *et al*[10]	79	F	Present (1.5)	Positive (Hp IgG-Ab)	Well	M	0-Is	Fundus	EMR	No recurrence for 3 yr
Johnson *et al*[11]	73	M	Present (2.4)	Positive (histology)	Well	SM	0-IIa + IIc	Corpus	TG	Unknown
Ozawa *et al*[3]	48	F	Present (1.5)	Negative (Hp IgG-Ab)	Poor	M	0-Is	Corpus to Antrum	TG	No recurrence for 2 yr
Charton-Bain *et al* [4]	62	F	Unknown	Negative (histology)	Well	M	0-Is	Corpus	TG	Death on the 7th day post-operation[2]
Present case	60	F	Present (3.4)	Negative (Hp IgG-Ab)	Well	M	0-Is	Corpus	ESD	No recurrence for 3 yr

[1]Hypoalbuminemia defined with less than 3.4 g/L.
[2]Death of pneumonia and liver failure.
EMR: Endoscopic mucosal resection; TG: Triglyceride; ESD: Endoscopic submucosal dissection; IgG: Immunoglobulin G.

unclear in one case.

These findings (protruding lesions located in the corpus or fundus region and hypoalbuminemia) may depict the clinicopathological features of Ménétrier's disease-associated early gastric cancer.

Ménétrier's disease generally presents with hypertrophic gastropathy, primarily in the fundic gland region, which may explain the tendency for Ménétrier's disease-associated early-stage gastric cancer to occur in this region[14]. Endoscopic gastric cancer screening of patients with Ménétrier's disease should focus on two characteristics: The location (corpus or fundus) and the macroscopic type.

In the present case, cancer could not be confirmed by preoperative biopsy; therefore, a definitive diagnosis of cancer was made using the specimen from the endoscopic resection specimen. Based on histopathological findings, Ménétrier's disease is considered a chronic inflammatory disease[15], making it difficult to differentiate between inflammatory atypia and neoplastic atypia *via* small tissue biopsy alone, as in the present case. When endoscopic biopsy tissue from a superficial tumor-like lesion exhibits atypical glands in patients with Ménétrier's disease, total biopsy with *en bloc* ESD can be useful for definitive diagnosis as well as treatment including complete curative resection, such as in this case.

Severe Ménétrier's disease cases with clinical symptoms such as abdominal pain and vomiting require total gastrectomy to control severe hypoalbuminemia[16,17]. Besides the present patient, the two other patients with *H. pylori*-negative early-stage cancer underwent total gastrectomy. There was one case of surgery-related death. Therefore, if Ménétrier's disease is not severe and the stomach can be preserved, ESD seems a beneficial therapy for early-stage gastric cancer, especially for mucosal cancer, because of its low invasiveness and high complete curative resection rate.

The mucin phenotype of the early gastric cancer in this case was gastric type with strongly positive for MUC5AC and low expression of MUC6. These suggest the gastric-type mucin of foveolar-dominant type. In the non-tumor area involved by Mén étrier's disease, although p53 staining was negative and Ki-67 index was low, significant hyperplasia of foveolar epithelium was shown in the fundic gland region. We deduced that early cancer in the present case could be developed from a polypoid lesion with foveolar hyperplasia along hyperplasia–dysplasia–carcinoma sequence [18], even though the mechanism of gastric cancer development in Ménétrier's disease is unknown.

CONCLUSION

To our knowledge, this is the first report of an extremely rare case of early-stage

gastric cancer detected in a patient with *H. pylori*-negative Ménétrier's disease in which complete curative resection was achieved with ESD.

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LETTER TO THE EDITOR

Artificial intelligence model validation before its application in clinical diagnosis assistance

Gustavo Jesus Vazquez-Zapien, Monica Maribel Mata-Miranda, Francisco Garibay-Gonzalez, Miguel Sanchez-Brito

ORCID number: Gustavo Jesus Vazquez-Zapien 0000-0002-6657-0722; Monica Maribel Mata-Miranda 0000-0002-1811-4511; Francisco Garibay-Gonzalez 0000-0002-1071-5208; Miguel Sanchez-Brito 0000-0003-4371-0486.

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Gustavo Jesus Vazquez-Zapien, Embryology Lab, Escuela Militar de Medicina, Ciudad de Mexico 11200, CDMX, Mexico

Monica Maribel Mata-Miranda, Cell & Tissue Biology Lab, Escuela Militar de Medicina, Ciudad de Mexico 11200, CDMX, Mexico

Francisco Garibay-Gonzalez, Department of Research, Escuela Militar de Medicina, Ciudad de Mexico 11200, CDMX, Mexico

Miguel Sanchez-Brito, Instituto Tecnológico de Zacatepec, Industrial Engineering, TecNM, Zacatepec 62780, Morelos, Mexico

Miguel Sanchez-Brito, Instituto Tecnológico de Aguascalientes, Computational Sciences, TecNM, Aguascalientes 20256, Mexico

Corresponding author: Miguel Sanchez-Brito, MD, PhD, Research Assistant Professor, Instituto Tecnológico de Zacatepec, Industrial Engineering, TecNM, Plan de Ayala, Zacatepec 62780, Morelos, Mexico. miguel.sb@zacatepec.tecnm.mx

Abstract

The process of selecting an artificial intelligence (AI) model to assist clinical diagnosis of a particular pathology and its validation tests is relevant since the values of accuracy, sensitivity and specificity may not reflect the behavior of the method in a real environment. Here, we provide helpful considerations to increase the success of using an AI model in clinical practice.

Key Words: Artificial intelligence; Diagnostic assistance; Validation tests; Leave-one-out cross-validation; K-fold validation; Hold-out validation

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Core tip: The validation tests and the process to adopt a particular artificial intelligence (AI) model are relevant. The percentages of accuracy, sensitivity and specificity obtained through validation techniques are strong indicators of whether the AI model is suitable for implementation in clinical practice or whether it will be necessary to

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continue acquiring samples.

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TO THE EDITOR

After studying the interesting article "Non-occlusive mesenteric ischemia: Diagnostic challenges and perspectives in the era of artificial intelligence" by Bourcier *et al*[1], who analyzed the current state of artificial intelligence (AI) in assisting clinical diagnosis and its possible application in diagnosing nonocclusive mesenteric ischemia, we are in full agreement with the AI techniques that the authors mention. However, a greater emphasis on the evaluation process for AI models could yield better results; when a rigorous testing stage is lacking, these models show poor performance upon transfer from the laboratory to real practice.

It is essential to mention that AI models using machine learning techniques, such as decision tree, support vector machine, artificial neural networks, naïve Bayesian classifier, Bayesian network K-nearest neighbor, and random forest, are predictive[2], are indispensable to performance of the three stages of training, validation and testing [3].

In this sense, the scarcity of validation tests provokes a reduction in the percentages of accuracy (Ac), sensitivity (Se) and specificity (Sp) of the AI models at the time of transferring them to a real environment[4].

These validation tests consist of segmenting the total of the samples available in different proportions to force the AI model to look for a robust solution (a representative pattern) due to the variance in the data. However, how to define the proportions in which the database will be segmented is a subject under development. Therefore, cross-validation strategies such as leave-one-out cross-validation (LOOCV) or k-fold cross-validation have been used more frequently than techniques such as hold-out validation because they obtain better Ac, Se and Sp in laboratory tests[4-6]; moreover, they consider a larger population in the training process compared to hold-out.

Following the LOOCV guidelines, a sample is left out of the database and the AI model is trained with the rest; once the training is finished, the separated spectrum is evaluated with the trained model. This process is repeated until all the collected spectra are evaluated, and the percentages of Ac, Se and Sp are calculated based on the number of correct and incorrect evaluations that the models have carried out. By involving most of the data in the training process, the result obtained by LOOCV usually reflects an overtrained model, making the generalization process of future samples complex by reducing their Ac, Se and Sp in a real scenario.

The k-fold model is similar to LOOCV, except that the database is divided into k groups with approximate numbers of samples instead of separating a single sample. Thus, one group is left out, and the rest is used for training; the process ends when all groups have been evaluated. The conflict with this strategy lies in defining the number of k groups created, since there is currently no formal methodology to calculate them. However, the most common values are k = 5 and 10; as such, the base data are segmented into five or 10 groups. In contrast, hold-out divides the populations that make up the database into percentages of 80–20 (one of the most used); that is, 80% of the samples from each population that make up the database are used for training. As this process is subjected to a more significant variance, the evaluation process usually shows lower percentages of Ac, Se and Sp compared to LOOCV and k-fold[4]. The above does not mean that the AI model is inadequate; instead, it indicates that the number of samples collected is insufficient to detect a sufficiently robust pattern. Thus, if the Ac, Se and Sp percentages are not reliable enough, acquiring more samples is a good option before using this AI methodology in clinical practice.

Although no studies have been carried out in this regard, an excellent strategy for evaluating whether an AI model is ready to be tested in a real environment is to

analyze several techniques, first using LOOCV and selecting the techniques with the best results to study their performance. Subsequently, k-fold evaluates the performance of the previously selected models thanks to the LOOCV strategy. As a result of the study of the models using the k-fold strategy, the model with the best performance should be selected. Finally, the best AI technique can be studied using the hold-out strategy; upon separating a considerable number of samples from each population according to the database (20%), the training/learning process of the AI models is subject to a more significant variance in the data of each population. In this way, they focus on particular features of the same group and not on characteristics of the samples that make up a particular database (overfitting), as could occur in the case of considering the LOOCV strategy only[4,5]. If the accuracy metrics of the model evaluated with hold-out are similar to those obtained when it was evaluated using LOOCV, it is possible to expect that the AI model will perform well in a real environment.

The use of AI methods in clinical diagnosis is new, and there are many subjects to investigate in this field; however, it is fascinating how the use of these technologies has reached medical science and how the new generations of researchers venture to use and combine the different sciences (physics, chemistry, mathematics, engineering, computer science, biology, among others) to generate new knowledge. We hope that the recommendations made here will help explore this AI field in the biological and medical sciences.

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LETTER TO THE EDITOR

Machine learning models and over-fitting considerations

Paris Charilaou, Robert Battat

ORCID number: Paris Charilaou 0000-0002-5512-4225; Robert Battat 0000-0002-7421-9764.

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Paris Charilaou, Robert Battat, Jill Roberts Center for Inflammatory Bowel Disease - Division of Gastroenterology & Hepatology, Weill Cornell Medicine, New York, NY 10021, United States

Corresponding author: Robert Battat, MD, Assistant Professor, Jill Roberts Center for Inflammatory Bowel Disease - Division of Gastroenterology & Hepatology, Weill Cornell Medicine, 1315 York Avenue, New York, NY 10021, United States. rob9175@med.cornell.edu

Abstract

Machine learning models may outperform traditional statistical regression algorithms for predicting clinical outcomes. Proper validation of building such models and tuning their underlying algorithms is necessary to avoid over-fitting and poor generalizability, which smaller datasets can be more prone to. In an effort to educate readers interested in artificial intelligence and model-building based on machine-learning algorithms, we outline important details on cross-validation techniques that can enhance the performance and generalizability of such models.

Key Words: Machine learning; Over-fitting; Cross-validation; Hyper-parameter tuning

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Core Tip: Machine learning models are increasingly being used in clinical medicine to predict outcomes. Proper validation techniques of these models are essential to avoid over-fitting and poor generalization on new data.

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TO THE EDITOR

Con *et al*[1] explore artificial intelligence (AI) in a classification problem of predicting biochemical remission of Crohn's disease at 12 mo post-induction with infliximab or adalimumab. They illustrate that, after applying appropriate machine learning (ML) methodologies, ML methods outperform conventional multivariable logistic

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regression (a statistical learning algorithm). The area-under-the-curve (AUC) was the chosen performance metric for comparison and cross-validation was performed.

Their study elucidates a few important points regarding the utilization of ML. First, the use of repeated k-fold cross-validation, which is primarily utilized to prevent over-fitting of the models. This technique, while common in ML, it has not been traditionally used in conventional regression models in the literature so far. Especially in small datasets, such as in their study (n = 146), linear (and non-linear, in the case of neural networks) relationships risk being "learned" by chance, leading to poor generalization of the models when applied to previously "unseen" or future data points. It was evident from their analysis that the "naïve" AUCs (training the model on all the data), was significantly higher than the mean cross-validated AUCs, in all 3 models, suggestive of "over-fitting" when one does not cross-validate. Smaller datasets tend to be more susceptible to over-fitting as they are less likely to accurately represent the population in question.

Second, the authors utilized "hyper-parameter tuning" for their neural network models, where the otherwise arbitrarily selected "settings" (or hyper-parameters, such as the number of inner neuron layers and number of neurons per layer) of the neural network are chosen based on performance. Hyper-parameters cannot be "learned" or "optimized" by simply fitting the model (as it happens with predictor coefficients), and the only way to discover the best values is by fitting the model with various combinations and assessing its performance. The combinations can be evaluated stochastically (randomly or *via* a Bayes-based approach) or using a grid approach (*e.g.*, for 3 hyper-parameters that take 5 potential values, there are $5 \times 5 \times 5 = 5^3 = 125$ combinations to evaluate) over k times. One may ask, if one was to fit a model 125 × k times, on 146 observations, is not there a risk for over-fitting the "optimal" hyper-parameter values? To avoid such a problem, nested k-fold cross-validation must be performed: within each repeated k-fold training data subset, a sub-k-fold "inner" training/validation must be done to evaluate each hyper-parameter combination. In this way, we overcome potential bias to optimistic model performance, which can occur when we use the same cross-validation procedure and dataset to both tune the hyper-parameters and evaluate the model's performance metrics (*e.g.*, AUC)[2]. The authors did not elaborate on how the hyperparameter tuning was performed.

Another point to consider in k-fold cross-validation in small datasets is the number of k-folds used, specifically in classification problems (*i.e.*, yes/no binary outcomes). In this study[1], the outcome prevalence was 64% ($n \approx 93$). With a chosen k = 5, the training folds would comprise 80% of that data, leading to approximately 74 positive cases of biochemical remission. The number of positive outcomes in each training fold must be considered, especially in logistic regression, where the rule of thumb recommends at least ten positive events per independent predictor, to minimize over-fitting[3]. In this study[1], six predictors were eventually used in the multivariable model, making over-fitting less likely from a model-specification standpoint. Finally, k-folds are recommended to be stratified by the outcome, so the outcome prevalence is equal among the training and testing folds. This becomes crucial when the prevalence of outcome of interest is < 10%-20% (imbalanced classification problem). While imbalanced classification is not an issue in this study[1], the authors did not mention whether they used outcome-stratified k-folds.

Lastly, the endpoint utilized, CRP normalization, has poor specificity for endoscopic inflammation in Crohn's disease[4]. More robust endpoints would include endoscopic inflammation and/or deep remission using validated disease activity indices[5].

We congratulate the authors for their effort, which acts both as a proof-of-concept for using ML in improved prediction of outcomes in IBD, but also for the methodologies outlined to reduce over-fitting. In general, with the advent of AI and specifically ML-based models in IBD[6], it is important to recognize that while now we have the tools to construct more accurate models and enhance precision medicine, most ML-based models, such as artificial neural networks, lack in being intuitively interpretable (*i.e.*, "black-box"). Efforts in "explainable AI" are under way[7], hopefully eliminating the "black-box" concept in future clinical decision tools. Applying these to validated disease activity assessments will be essential for prediction models in future studies.

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Contents

Weekly Volume 28 Number 6 February 14, 2022

OPINION REVIEW

608 Is precision medicine for colorectal liver metastases still a utopia? New perspectives by modern biomarkers, radiomics, and artificial intelligence

Viganò L, Jayakody Arachchige VS, Fiz F

MINIREVIEWS

624 Recent advances in the diagnostic evaluation of pancreatic cystic lesions

Ardeshna DR, Cao T, Rodgers B, Onongaya C, Jones D, Chen W, Koay EJ, Krishna SG

ORIGINAL ARTICLE

Basic Study

635 Effects of viremia and CD4 recovery on gut "microbiome-immunity" axis in treatment-naïve HIV-1-infected patients undergoing antiretroviral therapy

Russo E, Nannini G, Sterrantino G, Kiros ST, Pilato VD, Coppi M, Baldi S, Niccolai E, Ricci F, Ramazzotti M, Pallecchi M, Lagi F, Rossolini GM, Bartoloni A, Bartolucci G, Amedei A

Case Control Study

653 Atrophic gastritis and gastric cancer tissue miRNome analysis reveal hsa-miR-129-1 and hsa-miR-196a as potential early diagnostic biomarkers

Varkalaite G, Vaitkeviciute E, Inciuraite R, Salteniene V, Juzenas S, Petkevicius V, Gudaityte R, Mickevicius A, Link A, Kupcinskas L, Leja M, Kupcinskas J, Skieceviciene J

Retrospective Study

665 Validation of the PAGE-B score to predict hepatocellular carcinoma risk in caucasian chronic hepatitis B patients on treatment

Gokcen P, Guzelbulut F, Adali G, Degirmenci Salturk AG, Ozturk O, Bahadir O, Kanatsiz E, Kiyak M, Ozdil K, Doganay HL

CASE REPORT

675 Gallbladder Burkitt's lymphoma mimicking gallbladder cancer: A case report

Hosoda K, Shimizu A, Kubota K, Notake T, Hayashi H, Yasukawa K, Umemura K, Kamachi A, Goto T, Tomida H, Yamazaki S, Narusawa Y, Asano N, Uehara T, Soejima Y

LETTER TO THE EDITOR

683 COVID-19, liver dysfunction and pathophysiology: A conceptual discussion

Gonçalves Júnior J

689 Comments on validation of conventional non-invasive fibrosis scoring systems in patients with metabolic associated fatty liver disease

Hong JG, Yan LJ, Li X, Yao SY, Su P, Li HC, Ding ZN, Wang DX, Dong ZR, Li T

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Naoki Hashimoto, MD, PhD, Professor, Surgeon, Department of Surgery, Kindai University, Osaka 577-8502, Japan. gojigen000@gmail.com

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OPINION REVIEW

Is precision medicine for colorectal liver metastases still a utopia? New perspectives by modern biomarkers, radiomics, and artificial intelligence

Luca Viganò, Visala S Jayakody Arachchige, Francesco Fiz

ORCID number: Luca Viganò 0000-0001-5798-5021; Visala S Jayakody Arachchige 0000-0003-0754-5516; Francesco Fiz 0000-0003-3932-1330.

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Luca Viganò, Visala S Jayakody Arachchige, Department of Hepatobiliary and General Surgery, IRCCS Humanitas Research Hospital, Rozzano 20089, MI, Italy

Luca Viganò, Visala S Jayakody Arachchige, Department of Biomedical Sciences, Humanitas University, Pieve Emanuele 20072, MI, Italy

Francesco Fiz, Nuclear Medicine, IRCCS Humanitas Research Hospital, Rozzano 20089, MI, Italy

Corresponding author: Luca Viganò, MD, PhD, Professor, Department of Hepatobiliary and General Surgery, IRCCS Humanitas Research Hospital, Via Manzoni 56, Rozzano 20089, MI, Italy. luca.vigano@hunimed.eu

Abstract

The management of patients with liver metastases from colorectal cancer is still debated. Several therapeutic options and treatment strategies are available for an extremely heterogeneous clinical scenario. Adequate prediction of patients' outcomes and of the effectiveness of chemotherapy and loco-regional treatments are crucial to reach a precision medicine approach. This has been an unmet need for a long time, but recent studies have opened new perspectives. New morphological biomarkers have been identified. The dynamic evaluation of the metastases across a time interval, with or without chemotherapy, provided a reliable assessment of the tumor biology. Genetics have been explored and, thanks to their strong association with prognosis, have the potential to drive treatment planning. The liver-tumor interface has been identified as one of the main determinants of tumor progression, and its components, in particular the immune infiltrate, are the focus of major research. Image mining and analyses provided new insights on tumor biology and are expected to have a relevant impact on clinical practice. Artificial intelligence is a further step forward. The present paper depicts the evolution of clinical decision-making for patients affected by colorectal liver metastases, facing modern biomarkers and innovative opportunities that will characterize the evolution of clinical research and practice in the next few years.

Key Words: Colorectal liver metastases; Biomarkers; Genetics; Immune infiltrate; Radiomics; Artificial Intelligence

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Core Tip: The management of patients with colorectal liver metastases is challenging because the choice among different therapeutic options and strategies is not supported by strong evidence. A precision medicine approach has been an unmet need for a long time, but recent studies have opened new perspectives. In this paper, we will discuss new morphological approaches to assess tumor biology, the promising data from genetic analyses, the raising clinical relevance of the liver-tumor interface, and the potentialities of advanced imaging analysis and artificial intelligence. These are the keys to reach an effective personalized treatment in the near future.

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INTRODUCTION

During the last decades, the surgeons and medical oncologists drove the multidisciplinary teams to the ambitious aim of curing patients with colorectal liver metastases [1]. Systemic therapy had a progressively increasing effectiveness[2,3]. To date, the median life expectancy of patients receiving state-of-the-art treatment exceeds 30 mo[1,2]. The new immunotherapies could further raise the bar. Liver surgery has been the game-changer: It rapidly became the standard thanks to its proven safety (mortality risk lower than 2%) and oncological effectiveness (actual 5- and 10-year survival rates of about 50% and 20%, respectively)[1,4-6]. All patients with technically resectable disease, sufficient future liver remnant volume, and disease control by chemotherapy are now considered for surgery[1,7]. The liver surgeons pursued aggressive indications and developed complex techniques to maximize the resectability rate, even considering liver transplantation in the most recent years[7-9]. However, this generated a paradox: We are now searching for criteria to identify patients that are technically resectable but do not benefit from surgery because of their unfavorable tumor biology (10%-15% of patients have an early recurrence and early cancer-related death after surgery)[10]. Finally, thermal ablation gained momentum. After having demonstrated its effectiveness in patients with hepatocellular carcinoma, radiofrequency and microwave ablation have been successfully applied to patients with colorectal liver metastases, achieving adequate disease control[11,12]. Percutaneous treatments are now even tested as alternative to surgery in randomized trials[13,14].

The management of such a complex scenario should rely on an adequate understanding of tumor biology and several decisions need for a precision medicine approach (*e.g.*, the identification of the most appropriate schedule of systemic therapy, the selection of candidates to surgery, the indication to perioperative chemotherapy, the timing of colorectal and hepatic surgery in patients with synchronous metastases, and the choice between surgery and ablation). However, a recent study demonstrated that hepatobiliary surgeons have a huge heterogeneity in the treatment planning and surgical indications, the choice among different options being almost a throw of the dice[15]. Reliable biomarkers are urgently needed to drive a patient-tailored evidence-based approach.

In 2012, we depicted an evolving scenario with some preliminary evidence[16]. Where do we stand almost a decade later? In the present paper, we will provide a critical overview of traditional biomarkers, new proposals, and future perspectives (Figure 1 and Table 1).

MORPHOLOGY: AN OUTDATED BIOMARKER?

The tumor morphology is still the basis of several clinical decisions. The tumor burden defines the resectability of patients, and, in resectable ones, the need for perioperative

Table 1 Characteristics of different biomarkers of colorectal liver metastases.

	Biomarker characteristics					
	Standardized	Reproducibility	Robustness (across series)	Early assessment	Reliability in prediction	(Potential) Clinical impact
Morphology and clinical data	d	e	c	e	b	c
Dynamic evaluation	d	e	e	b	d	e
Genetics	c	d	d	e	e	e
Peritumoral tissue data	c	d	c	a	d	d
Radiomics	b	c	c	e	c	d
Artificial intelligence	a	a	b	d	d	e

The performances of every biomarker are evaluated by a score, ranging from "a" if very low to "e" if very high.

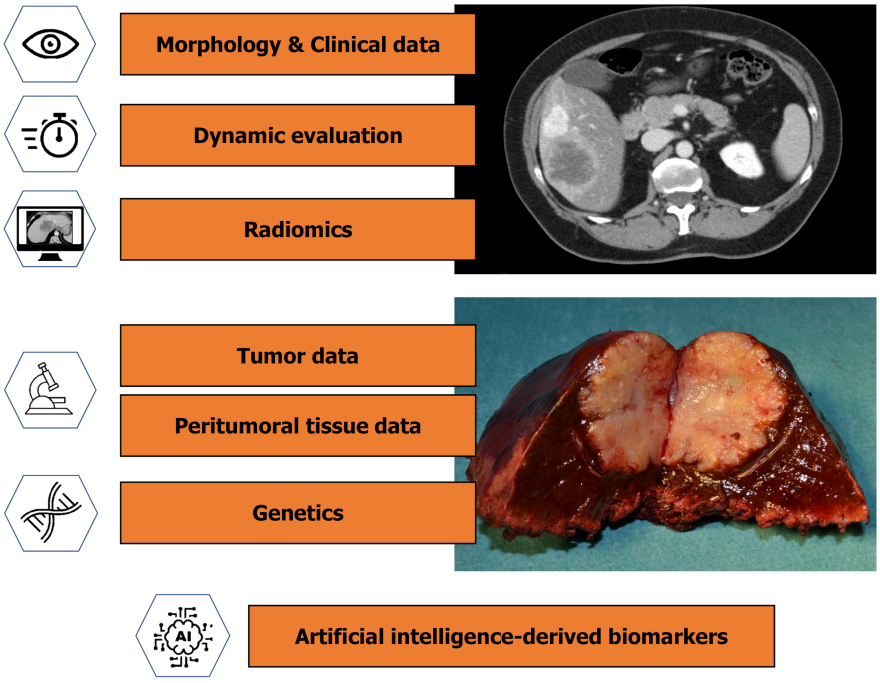


Figure 1 Available biomarkers for patients affected by colorectal liver metastases. A biomarker is defined as any parameter (molecular, cellular, clinical, imaging or identified by an artificial intelligence process) having a clinical role in narrowing or guiding treatment decisions and contributing to the estimation of the overall patient prognosis (prognostic biomarker), the clinical outcome after a treatment (predictive biomarker), or the properties of a clinical condition /disease (diagnostic biomarker).

chemotherapy[1,7]. The size of liver metastases determines the indication to thermal ablation (effective in nodules ≤ 30 mm)[17]. Several morphological parameters, including primary tumor data and tumor markers, have a prognostic value, and they have been combined into multiple scores to optimize their prognostic performance (Table 2)[18-23].

Recent studies reaffirmed the role of tumor morphology as a biomarker and determinant of the treatment strategy. First, Sasaki *et al*[24] proposed to combine the number and size of metastases into a "Tumor Burden Score", mimicking the Metroticket evidence for hepatocellular carcinoma[25]. They classified the patients into three groups and achieved a good stratification of survival, better than the stratification achieved by the size or the number of metastases when separately considered. Nevertheless, the Tumor Burden Score failed to select the candidates to surgery, the patients of the high-risk group (score ≥ 9) having an expected 5-year survival rate over 20%. Second, the primary tumor site has gained momentum. In comparison with patients having a left colonic tumor, those having a right colonic tumor are characterized by a lower response to chemotherapy, survival after surgery, and effectiveness

Table 2 Some of the available scores for outcome prediction of patients with colorectal liver metastases candidates to surgery

	Morphology-based scores				Morphology- and Genetics-based scores		
	Nordlinger *et al*[18], 1996	CRS, Fong *et al*[19], 1999	Iwatsuki *et al*[20], 1999	Rees *et al*[21], 2008	RAS Mutation CRS, Brudvik *et al*[60] 2017	GAME score, Margonis *et al*[61] 2018	Extended CRS, Lang *et al*[65], 2019
Morphological parameters							
Age	Yes (60 yr)						
Primary tumor							
Extension into the serosa	Yes						
N status primary tumor	Yes	Yes		Yes	Yes	Yes	Yes
Grading primary tumor				Yes			
Liver metastases							
Number	Yes (3)	Yes (1)	Yes (2)	Yes (3)		Yes (TBS)	
Size	Yes (50 mm)	Yes (50 mm)	Yes (80 mm)	Yes (50 mm)	Yes (50 mm)		Yes (50 mm)
Bilobar			Y				
DFI	Yes (24 mo)	Yes (12 mo)	Yes (30 mo)				
Surgical margin	Yes (10 mm)						
Extrahepatic disease				Yes		Yes	
CEA value		Yes (200 ng/mL)		Yes (60 ng/mL)		Yes (20 ng/mL)	
Genetic parameters							
RAS					Yes	Yes[1]	
RAS/RAF pathway							Yes
SMAD							Yes

[1]KRAS status.

DFI: Disease-free interval from primary to metastases; CEA: Carcinoembryonic antigen; CRS: Clinical risk score; GAME: Genetic and morphological evaluation; TBS: Tumor Burden Score.

of thermal ablation[26-29]. The embryological origin of the two parts of the colon (midgut for the right colon and hindgut for the left one) and the different genetic profiles of the tumors could explain such results. However, the impact of the primary tumor side on the treatment strategy is still to be defined, and, in this distinction (right *vs* left colonic cancer), the rectal cancers remain a blurred entity to elucidate. Third, a recent study based on the LiverMetSurvey data suggested that patients with synchronous multiple bilobar metastases should undergo a liver-first approach because this strategy achieves better survival than the alternative ones (*i.e.* the simultaneous and primary tumor-first approaches)[30]. This evidence could lead to a major change in current practice and definitively prioritizes the treatment of liver metastases in presence of a severe hepatic tumor burden. Fourth, in patients with liver and lung metastases, the pulmonary disease has shown a limited prognostic relevance [31]. Such data should be paired with those provided by Viganò *et al*[32], who demonstrated that the pathological response of colorectal metastases to systemic therapy changes according to the involved organ, being low in the lung and lymph nodes metastases, intermediate in the hepatic ones, and high in the peritoneal ones. The inhomogeneous prognostic relevance and chemosensitivity of the different tumor sites open new perspectives in treatment strategies and oncological research.

Despite its extensive adoption in current practice, tumor morphology is not a robust biomarker for several reasons. First, in patients undergoing systemic therapy,

morphology gives a limited prediction of the response to treatment. Second, in resectable patients, it does not allow for an adequate selection of candidates. The number of colorectal metastases and the presence of extrahepatic disease are paradigmatic examples. Even if the number of nodules is a strong prognostic factor, there is not a numeric cut-off value beyond which resection is contraindicated, and some patients with numerous metastases may benefit from surgery[33-35]. Similarly, the presence of extrahepatic disease contraindicates surgery in a limited proportion of patients (unresectable lesions, distant lymph node metastases, and diffuse peritoneal disease combined with multiple hepatic metastases)[36-38]. Third, different morphological parameters have been reported by different studies, and none has been confirmed by all authors. Fourth, morphological criteria can be modified by chemotherapy (*e.g.*, the tumor size), and it is unclear which value (before or after treatment) should be considered. Finally, tumor morphology offers a snapshot of the tumor and misses its evolution.

MOVING TOWARD A DYNAMIC VIEW

The tumor behavior is intuitively an effective surrogate biomarker of its biology. In the early 2000s, some authors proposed to adopt a time-test before surgery in patients with resectable colorectal liver metastases (*i.e.* an observation period to evaluate the tumor evolution)[39-41]. One-third to half of the patients developed additional lesions during the time-test and were excluded from resection. This policy has been early abandoned because of the advent of effective chemotherapy regimens, which combine observation and treatment. To date, neoadjuvant systemic therapy is a standard, and the tumor behavior during treatment is one of the most powerful prognostic factors. Since 2004, progression while on chemotherapy is even considered a contraindication to resection in resectable patients with few exceptions[42].

The prognostic role of the response to chemotherapy is indisputable, but three main limitations of this parameter should be highlighted: It excludes from surgery less than 10% of candidates[43]; the pathological evaluation of response has a poor agreement with the radiological one (about one-third of responders at imaging has no tumor regression at the pathology analysis)[44,45]; the no-progression during short chemotherapy (2-3 mo, the present standard) does not necessarily correspond to favorable biology and prognosis (about 15% of patients develop early recurrence after surgery)[10].

There is another time interval during which the tumor behavior can be analyzed. Patients must respect a 4-wk pause between the end of the systemic therapy and surgery (6 wk in case of anti-vascular endothelial growth factor treatment)[46,47]. We observed that about 15% of patients with tumor response or stabilization during chemotherapy have an early tumor progression in the interval between chemotherapy and surgery and an extremely poor outcome (0% survival at 2 years)[48]. Such a progression should contraindicate resection and dictates the need for restaging immediately before surgery.

Finally, percutaneous thermal ablation could contribute to the dynamic evaluation of colorectal liver metastases. It has been proposed as a time-test in patients with a synchronous disease or early recurrence after liver surgery with several benefits: Ablation provided a minimally invasive and effective treatment of the metastases, with high salvageability in case of local failure; avoided futile surgery in some cases; and spared chemotherapy for further disease progression[49,50].

Despite its effectiveness, the dynamic evaluation of colorectal metastases should be applied with caution. First, the time-test must be adequate. Progression during prolonged systemic therapy or after a long chemotherapy-surgery interval represents a loss of chance for resectable patients rather than a selection[48]. Even a disease progression in the interval between the two stages of a staged hepatectomy should not be considered *tout-court* an adequate selection of candidates[51]. Second, selected patients with a dimensional-only progression of the tumor and a limited hepatic tumor burden can be considered for surgery despite progression[52]. Finally, progression is not a definitive contraindication to resection, and surgery can be scheduled if the disease is controlled by a further line of chemotherapy[53,54].

GENETIC DATA: THE PANACEA FOR ALL THE UNCERTAINTIES?

Tumor genetics is the key to design a precision medicine approach. The sequencing of large series of metastases highlighted few high-frequency mutations, which have been extensively investigated for their association with the outcome. Tumor protein p53 (TP53) and APC gene mutations are the commonest ones (65%-75% and 45%-85% of patients, respectively)[55,56], but most studies focused on the RAS genes. KRAS and NRAS mutations are evident in one-third to half of the patients and have an established clinical impact: They preclude anti-epidermal growth factor receptor treatments and are associated with a lower response rate to chemotherapy, poorer survival, and higher risk of pulmonary metastases[57-59]. RAS status has been recently included in two prognostic scores for patients undergoing liver surgery (Table 2): The RAS Mutation Clinical Risk Score that considers the RAS status, metastases size, and N status of the primary tumor[60]; the Genetic And Morphological Evaluation (GAME) score that considers the KRAS status, carcinoembryonic antigen level, N status of the primary tumor, Tumor Burden Score, and presence of extrahepatic disease[61]. Both have been externally validated and outperformed the standard morphology-based scores. The patients with the highest scores had extremely poor outcome (0% recurrence-free survival at 2 years after surgery if RAS Mutation Clinical Risk Score = 3 or GAME score ≥ 6), but they were a marginal part of the cohort (14/564, 2.5%, and 18/1249, 1.4%, respectively).

The analysis of BRAF mutations generated a major interest despite their low frequency (4%-10%)[56,62]. The oncologists reported extremely poor survival of BRAF mutated patients, raising doubts about their candidacy to surgery[57,62]. Nevertheless, surgical series achieved an adequate outcome in selected BRAF mutated patients, suggesting that this genetic profile is a strong prognostic factor but should not be an absolute contraindication when the disease is adequately controlled by chemotherapy [63,64]. Additional mutations have been associated with prognosis, such as those of the TP53, PIK3CA, APC, and SMAD genes[65]. The Mainz group suggested that the performances of the aforementioned RAS score can be improved by replacing the RAS with the RAS-RAF pathway and adding the SMAD family (Table 2)[65]. The patients with all four negative prognostic factors (metastasis size > 50 mm, N+ primary tumors, and double mutation of the RAS-RAF pathway and SMAD family) had an extremely low median survival (1 year after surgery), but they were very few (only 5 out of 123, 4%). The MD Anderson Cancer Center group reported a cumulative negative prognostic impact of the mutations of TP53, RAS, and SMAD4: Survival progressively decreased with the increase in the number of the altered genes[66].

Those are the first steps of genetic-based precision medicine, but we have still to face some major challenges: Evidence is preliminary and needs robust validation to drive clinical practice; some criteria to select the candidates to surgery have been proposed, but they concern a minimal proportion of patients (< 5%)[60,61,65]; the discordance of the genetic profile between the primary tumor and metastases and their corresponding prognostic impact remain to be elucidated; tumor heterogeneity may lead to clonal populations with different mutations into a single metastasis, but their assessment is not yet standardized.

THE SOLUTION COULD BE OUTSIDE THE TUMOR

The liver-tumor interface could be the true battlefield where the interaction between the neoplastic cells and the "host" determines the prognosis. Several data are in favor of this hypothesis.

First, the pathology analysis of the peritumoral parenchyma highlighted the presence of the micrometastases (*i.e.* vascular and lymphatic tumoral emboli, perineural tissue infiltration, and satellite nodules)[44]. They are mainly localized within the first 2 mm of tissue surrounding the tumor, are reduced by chemotherapy, and negatively impact prognosis[44,67,68]. Micrometastases are the true determinants of the local recurrence risk after resection and thermal ablation.

Second, the profile of liver metastases has prognostic relevance. In 2009, Mentha *et al*[69] depicted the so-called "dangerous-halo" (*i.e.* a neoplastic regrowth at the tumor periphery due to an early reactivation of the metastases after the end of chemotherapy). This could represent the pathology counterpart of the radiological tumor progression that we observed in the interval chemotherapy-surgery. To date, the metastases' profile has been named "tumor growth pattern" and has been distinguished into three types: Pushing, desmoplastic, and replacement[70]. The types

correspond to different growing modalities: The metastases with a replacement pattern grow by co-opting the stroma and sinusoids; those with a pushing pattern have signs of active hypoxia-induced angiogenesis[71,72]. The replacement pattern is the most aggressive one and is associated with a lower response rate to chemotherapy, higher recurrence risk, and poorer survival[73-75]. In patients with a replacement pattern, we also observed an increased risk of local recurrence after surgery and the need for a wider surgical margin (unpublished data).

Third, a growing interest concerns the peri-tumoral immune infiltrate, especially after the introduction of modern immunotherapies. As for the primary colorectal cancers, an immunoscore, based on the presence of $CD3^+$ and $CD8^+$ cells in the core of liver metastases and at their invasion margin, achieved a good stratification of prognosis[76]. Additional cell populations have been investigated for their association with the outcome, such as the macrophages[77], but data are still preliminary.

Unfortunately, the biomarkers of the liver-tumor interface can be assessed only by the pathologist on the surgical specimen. The lack of an adequate non-invasive evaluation strongly reduces their clinical relevance. In addition, a comprehensive overview of the liver-tumor interface, merging the different pathology details, is still lacking, precluding a definitive understanding of the tumor-host interaction.

A further aspect deserves consideration; some features of the non-tumoral liver parenchyma could impact prognosis. Chemotherapy-associated sinusoidal injuries have been associated with the tumor response to chemotherapy; the more severe the sinusoidal dilatation the lower the response rate[44,78]. Nevertheless, the response to therapy and not the sinusoidal dilatation impacted survival[44]. In contrast, Viganò *et al*[44] depicted moderate/severe steatosis as a positive prognostic factor after surgery (5-year survival rate 53% *vs* 35%). These results have been confirmed by a subsequent analysis of the LiverMetSurvey database[79] and are in line with some studies reporting a favorable association between body mass index and prognosis[80,81]. We are still far from conclusive evidence and reliable explanation, but further investigations should be performed to potentially outline new therapeutic approaches.

RADIOMICS: IMAGING BEYOND THE VISIBLE DATA

Radiomics, or texture analysis, uses mathematical formulas to extract from medical imaging modalities invisible-to-the-eye patterns, which correlate with the biological properties of the analyzed tissue[82,83]. The complexity of analyses progressively increased, moving from histogram-based values to different types of matrices, filters, and transforms[84,85]. In patients with colorectal liver metastases, several potential applications of radiomics have been proposed[86]. First, it can predict the effectiveness of chemotherapy[87-95]. The decrease in entropy and increase in homogeneity of liver lesions after chemotherapy have been associated with the radiological tumor response. Some authors even reported the possibility to predict response to systemic therapy by analyzing the images at diagnosis before chemotherapy; higher entropy and lower homogeneity of liver metastases were associated with a subsequent higher response rate. When compared with the standard RECIST criteria, texture analysis achieved earlier and more accurate prediction. Second, radiomics have been associated with patients' prognosis, metastases with higher entropy and lower homogeneity having a better survival[88,90,96]. The comparative analysis of the imaging modalities before and after chemotherapy further refined the prediction of the long-term outcome[89,91,92,94], and there is accumulating evidence that both radiomic scores and combined clinical-radiomic models outperform traditional predictors of survival[92]. Third, textural features of the tumor before thermal ablation can predict the risk of local recurrence[97]. Fourth, radiomics are associated with the pathology data (*e.g.*, tumor grading, growth pattern, and regression grade after chemotherapy[88,98,99]). Finally, texture analysis has the potential to provide a non-invasive evaluation of the chemotherapy-associated liver injuries, which at present are poorly evaluated by standard imaging modalities[46,100].

The strength of radiomics relies on its capability to provide early prediction of the outcome and to reach a non-invasive estimation of the pathology details of colorectal metastases, anticipating data that are usually collected only after surgery. Further, the possibility to interpret the biological value of some radiomic features facilitates their implementation into clinical practice. For instance, entropy and heterogeneity, especially after contrast enhancement, clearly suggest the presence of active disease with heterogeneous clones, while homogeneity after chemotherapy reflects tumor necrosis due to a response to treatment. Finally, the development of technological tools

to perform automatic segmentation of liver tumors will enable easier extraction of radiomic features, contributing to the spread of such data. However, the texture analysis suffers from some limitations: Some features, in particular the second-order ones, lack interpretability; radiomics has instability across different devices and acquisition protocols, especially for magnetic resonance images; studies differ in terms of software packages, analyzed phases, and reported features; and reliable cut-off values of radiomic parameters are lacking. Those issues have to be solved to speed up the application of radiomics into clinical practice.

ARTIFICIAL INTELLIGENCE: WHERE DO WE STAND?

In the most recent years, the so-called "artificial intelligence" (AI) is the object of major interest and investments, with a consequent spike of AI-related publications[101]. Introduced in the 1950s, the term AI defines a computer program that, in a very specific setting, can "learn" and self-improve over time[102,103]. A demonstration of its potentialities took place in 1997, when a chess-playing AI, named Deep Blue, was able to beat the world champion Kasparov[104]. In medicine, AI is expected not only to optimize the prediction of an outcome by combining all available variables but also to update and improve continuously prediction according to the experienced results (Figure 2). AI can represent a major support to the decision-making processes, especially in the clinical scenarios with several therapeutic and strategical options and lack of consensus among experts, exactly as occurs for colorectal metastases[15]. In this sense, AI is not *per se* a biomarker but maximizes the profitability of all available data. However, AI may also have an additional role. It can be applied to medical imaging to identify new patterns that can contribute to diagnosis or prediction[105]. Such patterns, extractable from any type of imaging modality in a completely unbiased and unsupervised way, can be considered AI-derived biomarkers, subject to clinical validation[106]. Analogously, AI can identify biomarkers from any source of data, including clinical charts, medical reports, and images scan.

A first attempt in using AI-based therapy guidance dates to 2005, when a decision matrix platform, named OncoSurge, was introduced to help clinicians deciding the best treatment of patients at first diagnosis of colorectal liver metastases (*i.e.* when the treatment planning has the greatest impact)[107]. This method was later validated against the multidisciplinary team meeting achieving an almost perfect agreement [108]. Since then, few studies have been published, but they outlined a progressive increase in AI performances[109-113]. The AI predicted the recurrence risk after surgery by taking into account clinical, pathology, and laboratory data[110,112]. The addition of radiomic features into the machine learning models further optimized and anticipated the prediction[109,111]. Wei *et al*[113] compared a clinical, radiomic, and AI-based model to predict response to first-line chemotherapy; the deep-learning model had the best results, outperforming not only the model based on clinical parameters but also the one including texture analysis.

So far, the AI implementation into everyday practice is a priority to fill the quantum leap toward personalized computer-assisted medicine and will probably become a standard for clinical decision-making in the near future. It will allow merging all biomarkers, from morphological criteria to radiomics and genetic ones, weighing their prognostic role. Nevertheless, some current limitations of AI should be kept in mind. First, it needs training on large datasets, as Deep Blue did analyzing data from millions of chess matches[104]. Big data are crucial, but their availability is still limited by legal constraints and privacy policies. Shared databases and advanced interlinked frameworks could be the starting point. Second, AI supports decisions and does not replace clinical judgment yet, but computer-derived recommendations could lead to some legal and insurance critical issues. Finally, several technical and technological obstacles currently relegate AI-based approaches to highly specialized centers into a research setting.

CONCLUSION

To date, we are still far from a solid precision medicine for patients affected by colorectal liver metastases because of the limited capability of the available biomarkers to predict survival, response to chemotherapy, and the effectiveness of loco-regional therapies. Nevertheless, major (r)evolutions are ongoing, and the clinical approach to patients with metastatic colorectal cancer is going to change in the near future. The

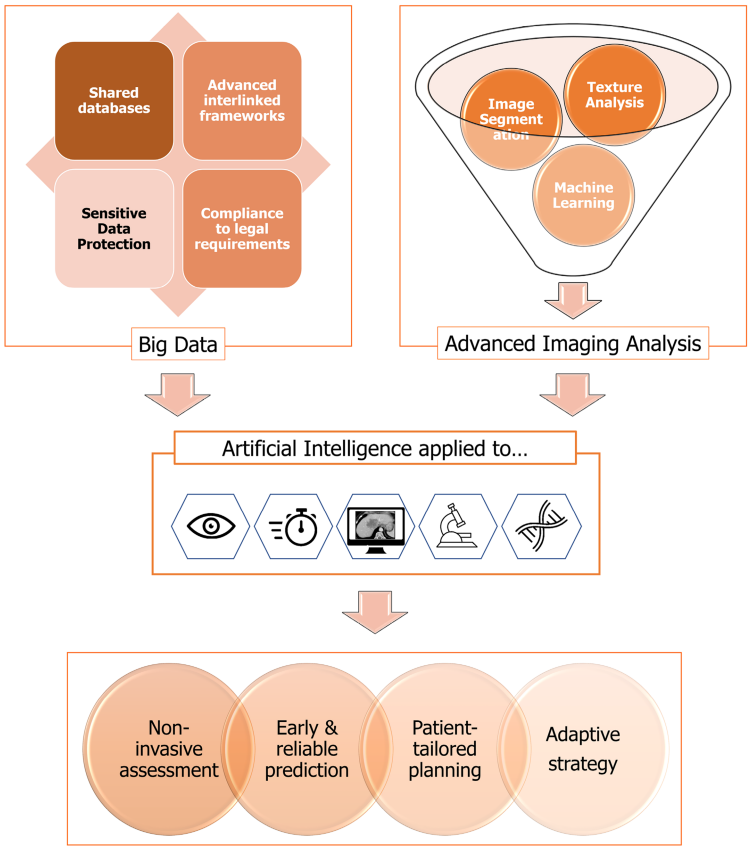


Figure 2 Future developments in the treatment planning for patients with colorectal liver metastases based on radiomics, big data, and artificial intelligence.

genetic analyses will definitively unveil the tumor biology, becoming the consistent basis of treatment planning; new biomarkers, based on radiomics and liver-tumor interface characteristics, will further enrich our comprehension and prediction of the tumor evolution; AI will merge and balance all data to drive decision-making processes.

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MINIREVIEWS

Recent advances in the diagnostic evaluation of pancreatic cystic lesions

Devarshi R Ardeshna, Troy Cao, Brandon Rodgers, Chidiebere Onongaya, Dan Jones, Wei Chen, Eugene J Koay, Somashekar G Krishna

ORCID number: Devarshi R Ardeshna 0000-0002-1849-0867; Troy Cao 0000-0003-3718-140X; Brandon Rodgers 0000-0002-6840-9676; Chidiebere Onongaya 0000-0003-2033-1440; Dan Jones 0000-0002-4688-3780; Wei Chen 0000-0002-9028-622X; Eugene J Koay 0000-0001-7675-3461; Somashekar G Krishna 0000-0001-5748-7890.

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Devarshi R Ardeshna, Chidiebere Onongaya, Department of Internal Medicine, Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Troy Cao, Brandon Rodgers, College of Medicine, Ohio State University, Columbus, OH 43210, United States

Dan Jones, James Molecular Laboratory, Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Wei Chen, Department of Pathology, Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Eugene J Koay, Department of GI Radiation Oncology, The University of Texas MD Anderson, Houston, TX77030, United States

Somashekar G Krishna, Division of Gastroenterology, Department of Internal Medicine, Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Corresponding author: Somashekar G Krishna, AGAF, FACG, FASGE, MD, Professor, Division of Gastroenterology, Department of Internal Medicine, Ohio State University Wexner Medical Center, 395 W. 12th Avenue, Suite 262, Columbus, OH 43210, United States. somashekar.krishna@osumc.edu

Abstract

Pancreatic cystic lesions (PCLs) are becoming more prevalent due to more frequent abdominal imaging and the increasing age of the general population. It has become crucial to identify these PCLs and subsequently risk stratify them to guide management. Given the high morbidity associated with pancreatic surgery, only those PCLs at high risk for malignancy should undergo such treatment. However, current diagnostic testing is suboptimal at accurately diagnosing and risk stratifying PCLs. Therefore, research has focused on developing new techniques for differentiating mucinous from non-mucinous PCLs and identifying high risk lesions for malignancy. Cross sectional imaging radiomics can potentially improve the predictive accuracy of primary risk stratification of PCLs at the time of detection to guide invasive testing. While cyst fluid glucose has reemerged as a potential biomarker, cyst fluid molecular markers have improved accuracy for identifying specific types of PCLs. Endoscopic ultrasound guided approaches such as confocal laser endomicroscopy and through the needle

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microforceps biopsy have shown a good correlation with histopathological findings and are evolving techniques for identifying and risk stratifying PCLs. While most of these recent diagnostics are only practiced at selective tertiary care centers, they hold a promise that management of PCLs will only get better in the future.

Key Words: Pancreatic cystic lesion; Intraductal papillary mucinous neoplasms; Mucinous cystic neoplasm; Microforceps biopsy; Radiomics; Confocal laser endomicroscopy

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Core Tip: Pancreatic cystic lesions (PCLs) are highly prevalent. It is critical to accurately diagnose PCLs and risk stratify them to guide management. Current diagnostic techniques are suboptimal; hence, recent investigations have focused on developing, refining, and validating novel technologies for accurately diagnosing specific cyst type and ascertaining high-risk lesions for malignancy. Radiomics, cyst-fluid biomarkers, confocal laser endomicroscopy and microforceps biopsy hold the promise of accurately diagnosing PCLs and improving their management.

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INTRODUCTION

Pancreatic cystic lesions (PCLs) are increasingly detected, largely due to advances in imaging techniques and the increasing age of the general population[1]. With prevalence estimated in the range of 4%-14% in the general population and increasing constantly, it has become essential to characterize and risk stratify these cysts to guide management[2]. Current guidelines for evaluating PCLs are limited to less than optimal diagnostic techniques, resulting in either missed detection of early cancer or surgical over-treatment (see Figure 1). Resection of PCLs should be extremely selective since pancreatic surgery generally has a 20%-40% morbidity rate and an approximate 2% mortality rate[3-5]. Therefore, research and utilization of safe and effective diagnostic modalities with high accuracy are needed to evaluate cysts and introduce properly timed interventions.

Addressing this issue is especially relevant for intraductal papillary mucinous neoplasms (IPMNs), a type of PCL with one of the highest risks for malignancy. Of the two IPMN subtypes, main duct IPMNs are reported to have a risk from 38% to 68% and branch duct IPMNs from 12% to 47%[6]. Substantial research has addressed the use of consensus guidelines for evaluating IPMNs, but all mention that significant areas of improvement is imperative[7-9].

Current standards for the evaluation of cyst morphology include computed tomography (CT) scan, magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS). Fine needle aspiration (FNA) of cyst fluid for carcinoembryonic antigen (CEA) and cytology is performed during EUS. Considerable heterogeneity exists among the five widely used guidelines, which indicate a lack of standardization in diagnostic workups[7-12]. In terms of the target population, American College of Gastroenterology and European guidelines include all PCLs, American College of Radiology guidelines focus on incidental PCLs, Fukuoka guidelines only focuses on IPMNs and American Gastroenterological Association (AGA) includes all PCLs except main-duct IPMNs. Guidelines differ in recommending evaluation with EUS and EUS-FNA, and surgical resection. Multiple studies have compared some of these guidelines for identifying high-risk PCLs. Amongst patients who underwent surgery, the current guidelines directed clinical decision with an accuracy, sensitivity and specificity of 49.6%, 23.5%, 84.3% for 2015 AGA guidelines, 41.2%, 39.7%, 43.1% for revised Fukuoka guidelines and 58%, 67.7%, 45.1% for 2018 European guidelines[13].

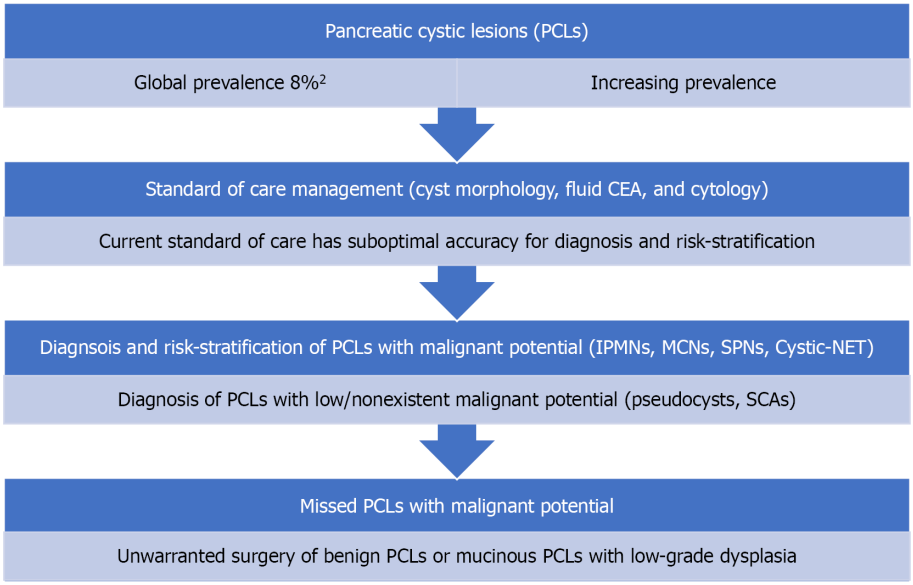


Figure 1 Current standard of care diagnostic methods are suboptimal in the diagnosis of specific types of pancreatic cystic lesions and risk-stratification of mucinous cysts. PCL: Pancreatic cystic lesion, CEA: Carcinoembryonic antigen, IPMN: Intraductal papillary mucinous neoplasms, MCN: Mucinous cystic neoplasm, SPN: Solid pseudopapillary neoplasm, Cystic-NET: Cystic neuroendocrine tumors, SCA: Serous cystadenoma.

A better understanding of investigational characteristics that lead to malignancy is necessary to improve existing criteria and accurately determine associated risks (Figure 1). While cyst fluid glucose has reemerged as a potential biomarker, novel techniques such as cyst fluid molecular analysis, EUS-guided needle-based confocal laser endomicroscopy (EUS-nCLE) and microforceps biopsy (EUS-MFB) have been introduced. The aim of this review is to provide an update of the recent literature in the management of PCLs with an emphasis on novel diagnostic methods.

RADIOMICS

Increasing prevalence of incidental PCLs has placed significant pressure on the necessity of discerning low-risk and high-risk lesions identified in radiological images. Radiomics is the analysis of mathematically derived textural features from cross-sectional imaging studies. The features are generally beyond human visual perception. Using radiometric feature extraction tool, radiomics can quantify individual pixels and their associated gray-scale value from cross-sectional imaging in a temporal and spatial plane to create a cyst impression. While studies have varied in the extraction of radiometric data, these features can potentially risk stratify PCLs. Hence, radiomics can guide downstream invasive diagnostics.

Studies in radiomics can be classified into two broad categories: (1) Differentiating types of PCLs, and (2) Risk stratification of IPMNs. Investigators have applied machine learning algorithms to radiomic features for automatic classification of PCLs. Some recent studies have evaluated nomograms and algorithms combining radiomics, cyst morphology, and clinical features. For differentiating PCLs, several investigations have demonstrated promising results. One of the first studies by Dmitriev *et al*[14], achieved a reasonable accuracy of 83.6% in discriminating PCL types into IPMNs, mucinous cystic neoplasms (MCNs), serous cystadenoma (SCAs) and solid neoplasms [14]. Their model had 93.2%-95.9% accuracy at predicting IPMNs. Subsequently, three investigations utilized CT-Scan radiomics to differentiate serous cystadenomas from other PCLs (area under the curve (AUC) 0.77-0.99, sensitivity 69%-95%, specificity 71%-96%)[15-17]. In one of these studies, radiomics outperformed radiologic characteristics in differentiating MCNs and macrocystic SCAs; comparative diagnostic parameters included sensitivity (93.6% *vs* 74.2%), specificity (96.2% *vs* 80.8%) and accuracy (94.7% *vs* 77.2%), respectively[16]. Combining radiomics with radiological findings or clinical parameters significantly improved the accuracy to distinguish cyst types in comparison to radiomics alone ($P < 0.05$) [16,18].

Only a few studies have evaluated the role of radiomics in differentiating IPMNs with advanced neoplasia, from indolent lesions with low-grade dysplasia (Table 1)[19-

Table 1 Summary of studies evaluating the role of radiomics in differentiating intraductal papillary mucinous neoplasms with advanced neoplasia

Ref.	*n*	Image type	No. of radiomic features	Best model	Performance training set
Hanania *et al*[19], United States, 2016	53	CECT	360	10 radiomic features	AUC: 0.82 SP: 85%, SP: 68%
Permuth *et al*[20], United States, 2016	38	CECT	112	14 radiomic features +blood 5 mi-RNAs	AUC: 0.92 SN: 83%, SP: 89%
Attiyeh *et al*[21], United States, 2019	103	CECT	255	Radiomic + clinical features	AUC: 0.79 SN: 71%, SP: 82%
Williams *et al*[22], United States, 2020	33	CECT	12	Radiomic features + cyst fluid protein markers	AUC: 0.88 SN: 71%, SP: 92%
Hoffman *et al*[23], United States, 2017	18	MRI w/ DWI	N/A	Entropy	AUC: 0.86 SN: 100%; SP: 70%

HGD: High-grade dysplasia; LGD: Low-grade dysplasia; CECT: Contrast-enhanced computed tomography; MRI: Magnetic resonance imaging; mi-RNA: micro-RNA; DWI: Diffusion weighted imaging; AUC: Area under curve; SN: Sensitivity; SP: Specificity; NA: Not application.

23]. Most of the studies evaluating radiomics in IPMNs have used CT scans, and included patients with confirmed surgical histopathology as ground truth. A recent study by Cui *et al*[24], presents the first publication where a radiomic signature incorporating 9 features was combined with clinical variables to predict high-grade dysplasia or adenocarcinoma (advanced neoplasia) in branch duct-IPMNs. Their predictive nomogram diagnosed advanced neoplasia with AUC values of 0.903 (training cohort; sensitivity 95%, specificity 73%), and 0.884 (one of two external validation cohorts; sensitivity 79%, specificity 90%)[24].

Thus, radiomics represents a promising non-invasive approach for the classification and risk stratification of PCLs and will favorably impact patient management. However, radiomics continues to be a novel concept and has been largely used to date in clinical trials at academic centers. While radiomics has demonstrated an immense potential for diagnosis, prognosis, and risk assessment in PCLs; there is a need for standardized protocols for image acquisition, segmentation, feature extraction, and analysis.

TRADITIONAL DIAGNOSTIC APPROACHES USING BIOMARKERS

Cyst fluid analysis

CEA and amylase: Traditionally pancreatic cyst fluid is aspirated using EUS-FNA for biomarker and cytologic analysis. In early studies, cyst fluid CEA levels above 192 ng/mL was shown to correlate with mucinous PCL with 79% (88/111) accuracy ($P < 0.0001$)[25]. However more recent studies, have estimated CEA sensitivity and specificity at 63% and 88%, respectively in differentiating mucinous from non-mucinous cysts[26]. This level of accuracy would result in misdiagnosis of 35%-39% of mucinous cysts. Additionally, CEA levels across sites are difficult to compare and levels have not been shown to correlate with PCL malignant potential[25,27-29]. Regarding amylase levels, a low cyst fluid amylase level has very high specificity for excluding pseudocyst. However, high amylase levels have been shown to have no diagnostic utility[26,30]. As a result, measuring amylase has fallen out of favor for the diagnosis of PCLs.

Cytology: Cyst fluid analysis by cytology has been shown to lack sensitivity for the diagnosis of PCLs. A meta-analysis with 937 patients demonstrated cyst fluid cytology to have 63% sensitivity and 88% specificity for the diagnosis of PCL[31]. Another meta-analysis calculated cytology to have 51% and 94% sensitivity and specificity, respectively[32]. This lack of sensitivity results from cytology evaluations usually detecting only intact exfoliated cells that are typically few in number[25,33].

Glucose: Intracystic glucose has good accuracy at differentiating mucinous and non-mucinous cysts but this economical diagnostic tool has not been used in routine clinical practice. However, recent prospective studies have provided improved and sustained evidence that cyst fluid glucose should be considered for standard of care evaluation of PCLs. Low intra-cystic glucose concentration is predictive of a mucinous cyst while high concentrations are consistent with non-mucinous cysts. In 2020, Ribaldone *et al*[34] reported from 56 patients that intra-cystic glucose concentration < 50 mg/dL had significantly better sensitivity than a CEA level > 192 ng/mL for diagnosing mucinous cysts (93.6% *vs* 54.8%; P = 0.003). Both CEA and intra-cystic glucose had high specificity for diagnosing mucinous cysts (96% *vs* 100%; P = 1). They reported that intra-cystic glucose concentration of more than 50 mg/dL had higher sensitivity than CEA values of less than 5 ng/mL for diagnosing non-mucinous cysts (96% *vs* 72%, P = 0.07).

A meta-analysis of 7 studies encompassing 566 patients reported that lower (cut-off < 50 mg/dL) intra-cystic glucose concentration had a pooled sensitivity of 90.1% (95%CI: 87.2-92.5) and pooled specificity of 85.3% (95%CI: 76.8-91.1) when differentiating mucinous from non-mucinous cysts[35]. In a subset analysis, point-of-care glucometer measurements for intra-cystic glucose (3 studies) also revealed comparable pooled sensitivity of 89.5% (95%CI: 85.5-92.5; I^2 = 0) and pooled specificity of 83.9% (95%CI: 68.5-92.6; I^2 = 43) for the differentiation of PCLs[35]. A more recent (2021) meta-analysis that included 8 studies with 609 PCLs showed pooled sensitivities for glucose *vs* CEA of 91% (95%CI: 88-94) *vs* 56% (95%CI 46-66) (comparative P value < 0.001), pooled specificities were 86% (95%CI: 81-90) *vs* 96% (95%CI: 90-99), P > 0.05, respectively[36].

Estimation of glucose levels is a low-cost diagnostic test that has repeatedly demonstrated better accuracy at differentiating mucinous and non-mucinous cyst. While not being definitive, cyst fluid glucose is a practical and economical diagnostic tool that can help in the differentiation of PCLs.

Molecular markers: With the introduction of next-generation sequencing (NGS), diagnosis of PCLs with either small gene panels and whole exome NGS have been employed. This method allows assessment of intact cell and cell-free nucleic acid that has been shed into the cyst fluid. DNA mutations that are commonly associated with pancreatic adenocarcinoma (*KRAS, CDKN2A, SMAD4, PTEN, PIK3CA,* and *TP53*) may also be present in precursor PCLs, with the latter five associated with advanced neoplasia.

Similar to radiomics, molecular analysis of cyst fluid can contribute to the classification of PCLs, and risk stratification of IPMNs. In a meta-analysis (6 studies, 785 PCLs), McCarty *et al*[37] reported that the dual presence of *KRAS* and *GNAS* mutations detected mucinous PCLs with a sensitivity of 75% (95%CI: 58-87%), specificity of 99% (95%CI: 67-100%), and diagnostic accuracy of 97% (95%CI: 95-98%), respectively. For specifically diagnosing IPMNs, dual *KRAS/GNAS* mutation had 94% (95%CI: 72-99%) sensitivity, 91% (95%CI: 72-98; I^2 = 89.83%) specificity and 97% (95%CI: 95-98%) accuracy, respectively. Recently, our group identified, for the first time, that uncommon *BRAF* mutations (and occasional *MAP2K1* mutations) characterize a significant subset of IPMNs that lack *KRAS* mutations, indicating that RAS-MAPK dysregulation is ubiquitous in these tumors[12]. In the same study, we showed 88.5% sensitivity, 100% specificity, and 90.3% accuracy for NGS differentiation of PCLs[12].

For the risk stratification of IPMNs, Singhi *et al*[38] used next-generation sequencing to evaluate DNA mutations associated with advanced neoplasia. In a subgroup analysis of 102 patients with histopathologic diagnosis, they reported that the presence of TP53, PIK3CA and/or PTEN mutation had 88% (95%CI: 62-98%) sensitivity and 95% (95%CI: 88-98%) specificity, respectively for diagnosing IPMNs with advanced neoplasia.

Cyst fluid molecular analysis by next generation sequencing is superior to measuring cyst CEA levels with superior accuracy and the ability to provide risk stratification for IPMNs. However, it is selectively available and represents a logistical and financial barrier for universal adaptation.

ADVANCED INTERVENTIONAL DIAGNOSTIC APPROACHES

EUS-guided needle confocal laser endomicroscopy

EUS-guided needle confocal laser endomicroscopy (nCLE) permits real-time microscopic imaging of intra-cystic epithelium within a single plane. It allows for *in*

vivo pathological analysis of PCLs. Early studies have established the characteristic features for IPMNs. Investigations by Napoleon *et al*[40] in the CONTACT study established defining criteria for MCNs, SCAs, and cystic neuroendocrine tumors[39, 40]. In 2020, the INDEX study provided further support for nCLE as a viable diagnostic tool by demonstrating high performance in differentiating PCLs amongst the highest number (n = 65) of patients with surgical histopathology[41]. For the differentiation of PCLs into mucinous and non-mucinous lesions, a recent meta-analysis with 7 studies and 324 patients reported a pooled sensitivity, specificity and accuracy of 85% (95%CI: 71-93%), 99% (95%CI: 90-100%) and 99% (95%CI: 98-100%), respectively. The pooled risk of post-procedure acute pancreatitis was 1% (95%CI: 0-3%)[42]. Another recent meta-analysis (10 studies, 536 patients) reported a pooled sensitivity, specificity, and accuracy of 82.4% (95%CI: 74.7-90.1%), 96.6% (95%CI: 94.3-99%), and 88.6% (95%CI: 83.7-93.4%), respectively, for the differentiation of mucinous from non-mucinous PCLs[43].

In addition to the high accuracy of diagnosing IPMNs and other cysts, nCLE can potentially determine the risk for advanced neoplasia in PCLs. To detect advanced neoplasia in IPMNs, multiple nCLE imaging variables were identified in a post-hoc analysis of the INDEX study[44], Figure 2. This study identified that the variables with the highest interobserver agreement were papillary epithelial thickness and darkness. Specifically, nCLE visualized papillary epithelial thickness (width ≥ 50 μm) had a sensitivity, specificity, and AUC of 87.5% (95%CI: 62%-99%), 100% (95%CI: 69%-100%), and 0.95, respectively for the detection of advanced neoplasia. Also, estimation of the papillary epithelial darkness (cut-off ≤ 90 pixel intensity) revealed a sensitivity, specificity, and AUC of 87.5% (95%CI: 62%-99%), 100% (95%CI: 69%-100%), and 0.90, respectively[44]. Analogously for mucinous cysts, Feng *et al*[45] reported that nCLE pattern of "dark aggregates of neoplastic cells" correlated with the morphologic features of "irregular branching and budding" and was diagnostic of malignancy, with 75% sensitivity, 100% specificity and 94% accuracy, respectively.

However, potential limitations of nCLE include differences in interobserver interpretation of images and the tedious nature of manually determining papillary epithelial thickness and darkness. Both of these issues were addressed with the development of a machine learning artificial intelligence model that identified advanced neoplasia in IPMNs with a sensitivity (83%) and specificity (88%) well above the Fukuoka or AGA guidelines[46].

Despite the growing evidence of nCLE as a viable diagnostic technique, its incorporation into standard clinical evaluation is lacking. The primary challenges include equipment costs, optimal training in image acquisition and interpretation, and prevention of adverse events higher than the standard EUS-FNA process.

EUS guided MFB or EUS-through-the-needle biopsy

This technique utilizes an EUS guided approach to pass a specialized device, the Moray micro forceps (Moray micro forceps, US Endoscopy, Mentor, Ohio, United States) through the 19-gauge EUS needle to collect tissue sample from PCLs. Multiple recent studies have demonstrated an improved diagnostic yield and accuracy in the diagnosis of specific types of PCLs[47,48].

Multiple meta-analyses have been published and the most recent studies include the following. Tacelli *et al*[49] (2020) included 9 studies with 454 patients and pooled technical success, histological accuracy and diagnostic yield for specific types of PCLs were 98.5% (95%CI: 97.3%-99.6%), 86.7% (95%CI: 80.1-93.4) and 69.5% (95%CI: 59.2-79.7%), respectively. Additionally sensitivity and specificity for diagnosis of mucinous PCLs were 88.6% and 94.7%, respectively. However, the overall complication rate was 8.6% (95%CI: 4.0-13.1%) with studies reporting rates ranging from 1%-23%. Of the reported complications, 57.1% had self-limiting bleedings (most commonly intra-cystic bleeding), 24.5% had mild pancreatitis, 6.1% had infections and 14.3% had abdominal pain. Westerveld *et al*[50] analyzed 8 studies with 426 patients reporting similar results. The MFB approach had significantly higher diagnostic yield for specific cyst type compared to cytology (72.5%, 95%CI: 60.6-83.0% *vs* 38.1%, 95%CI: 18.0-60.5%). Additionally, MFB had significantly higher diagnostic yield for mucinous cyst compared to cytology (OR: 3.86; 95%CI: 2.0-7.44, I^2 = 72%). Overall MFB procedures had a 7% complication rate with 5% incidence of intra-cystic hemorrhage and 2.3% risk of acute pancreatitis. More importantly, in a subgroup analysis of 92 patients who had surgical resection of their PCLs, MFB findings had concordance of 82.3% (95%CI: 71.9-90.7%) for specific cyst diagnosis. MFB findings for mucinous cysts had a sensitivity of 90.1% (95%CI: 78.4-97.6%) and specificity of 94% (95%CI: 81.5-99.7%). Additionally, the concordance rate for histological grade of dysplasia was 75.6% (95%CI: 62.3-86.8).

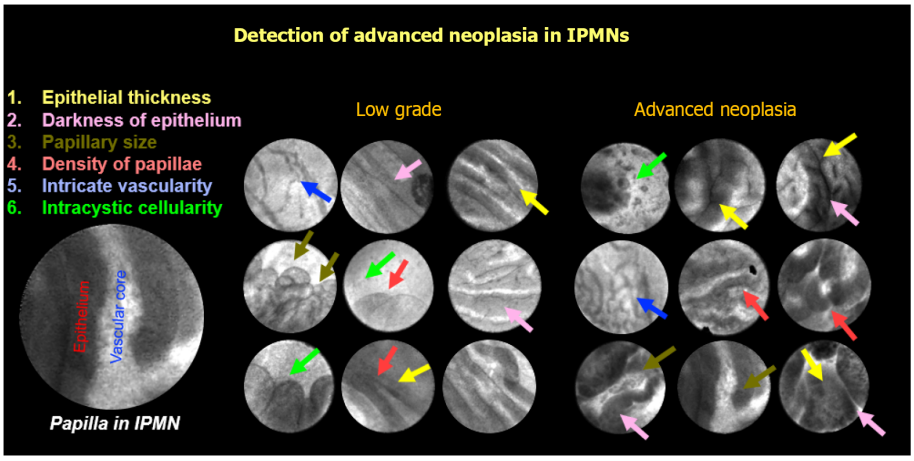


Figure 2 Features identified on endoscopic ultrasound guided needle confocal laser endomicroscopy. IPMN: Intraductal papillary mucinous neoplasms.

In another meta-analysis that included patients with surgical histopathology as reference diagnosis, the pooled sensitivity and specificity for diagnosing mucinous PCL was 86% (95 %CI: 62-96%) and specificity 95% (95%CI: 79-99%) respectively[51]. For diagnosis of specific cyst type, the pooled sensitivity and specificity were 69% (95%CI: 50-83%) and specificity 47% (95%CI: 28-68%), respectively. The authors also grouped IPMNs and MCNs with advanced neoplasia, SPNs, and cystic neuroendocrine tumors as high-risk cysts. MFB demonstrated a pooled sensitivity and specificity of 78% (95%CI: 61-89%) and 99% (95%CI: 90-99%) respectively for diagnosis of a high-risk cyst.

While MFB represents an excellent technique for acquisition of tissue and accurately diagnosing PCLs, the high rates of adverse events including acute pancreatitis and intra-cystic bleeding may deter clinicians from using this technique.

Contrast-enhanced EUS

EUS when combined with contrast enhancers allows detection of vascularity within PCLs. This allowed contrast-enhanced EUS (CE-EUS) to differentiate pseudocysts from true PCLs and identify mural nodules within PCLs. Despite early studies reporting no improvement over traditional EUS at differentiating PCLs[52], recent studies have reported higher diagnostic yield for PCLs using CE-EUS (96% compared to 71% for traditional EUS)[53]. CE-EUS detected small lesions initially missed on contrast-enhanced CT or EUS-FNA[54]. Recent literature on CE-EUS has reported higher accuracy at diagnosing PCLs compared to CT, MRI and traditional EUS[55]. Despite these encouraging results, CE-EUS has not gained traction in clinical management of PCLs.

CONCLUSION

Future directions in the diagnosis of pancreatic cysts

Reliable and accurate diagnosis of PCLs is a bottleneck for appropriate management of these lesions. Although, novel diagnostics have improved the diagnostic accuracy, there is still a dearth of prospective multicenter studies and a need to understand the complementary role of these tests. Radiomics, as a non-invasive tool has the potential for preliminary risk stratification of PCLs into low-and-high risk lesions (Figure 3). The technique holds a potential to allow clinicians to skip expensive and invasive diagnostic techniques on certain low risk PCLs.

For low-risk PCLs, and when EUS-FNA is indicated, low-cost cyst fluid analysis with glucose, CEA, and cytology can guide management (Figure 3). If radiomics and EUS cyst morphology are indicative of a high-risk PCL, advanced diagnostics with cyst fluid molecular analysis, nCLE, or microforceps biopsy can be considered based on the center and endoscopists' expertise. The rate of adverse events with microforceps biopsy needs to be considered when considering this test.

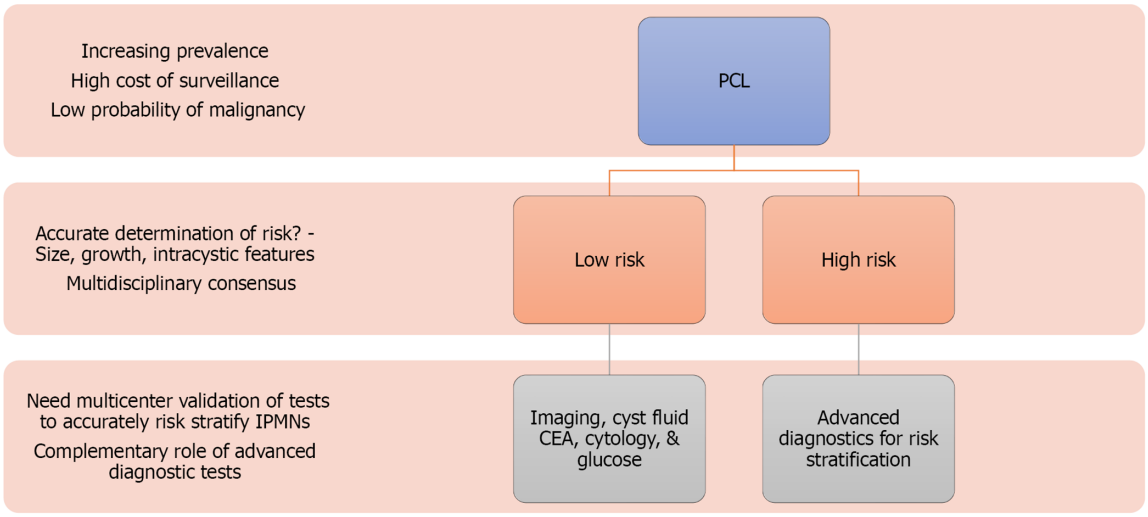


Figure 3 Future directions of detection and risk stratification of pancreatic cystic lesion to guide clinical management. PCL: Pancreatic cystic lesion, CEA: Carcinoembryonic antigen, IPMN: Intraductal papillary mucinous neoplasms.

Despite the availability of multiple diagnostic methods, the diagnosis and management of PCLs continues to be challenging. The more recent diagnostic modalities lack supportive larger multicenter data and there is need to demonstrate cost-effectiveness when compared to using suboptimal techniques and resultant unwarranted resection of otherwise benign or indolent PCLs. Apart from diagnosis, surveillance methods for low-risk lesions needs innovation as current tools are resource-consumptive.

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ORIGINAL ARTICLE

Basic Study

Effects of viremia and CD4 recovery on gut "microbiome-immunity" axis in treatment-naïve HIV-1-infected patients undergoing antiretroviral therapy

Edda Russo, Giulia Nannini, Gaetana Sterrantino, Seble Tekle Kiros, Vincenzo Di Pilato, Marco Coppi, Simone Baldi, Elena Niccolai, Federica Ricci, Matteo Ramazzotti, Marco Pallecchi, Filippo Lagi, Gian Maria Rossolini, Alessandro Bartoloni, Gianluca Bartolucci, Amedeo Amedei

ORCID number: Edda Russo 0000-0003-3141-1091; Giulia Nannini 0000-0002-6481-6864; Gaetana Sterrantino 0000-0002-0385-0903; Seble Tekle Kiros 0000-0002-9687-0734; Vincenzo Di Pilato 0000-0002-5863-5805; Marco Coppi 0000-0002-4387-4037; Simone Baldi 0000-0002-5151-2618; Elena Niccolai 0000-0002-9205-8079; Federica Ricci 0000-0002-9986-7039; Matteo Ramazzotti 0000-0002-6862-9409; Marco Pallecchi 0000-0002-0057-6531; Filippo Lagi 0000-0003-0999-6070; Gian Maria Rossolini 0000-0002-9386-0434; Alessandro Bartoloni 0000-0001-9758-1523; Gianluca Bartolucci 0000-0002-5631-8769; Amedeo Amedei 0000-0002-6797-9343.

Author contributions: Russo E and Nannini G contributed equally to the work; Sterrantino G, Russo E, and Amedei A designed the study; Russo E and Sterrantino G revised the literature on this topic; Tekle Kiros S, Niccolai E, Ricci F, Russo E, Baldi S, and Nannini G collected the data; Russo E, Coppi M, Di Pilato V, Nannini G, Baldi S, Ricci F, and Lagi F analysed the data; Ramazzotti M and Di Pilato V performed microbiota analysis; Russo E wrote the manuscript; Russo E edited the manuscript;

Edda Russo, Giulia Nannini, Gaetana Sterrantino, Seble Tekle Kiros, Marco Coppi, Simone Baldi, Elena Niccolai, Federica Ricci, Filippo Lagi, Alessandro Bartoloni, Amedeo Amedei, Department of Clinical and Experimental Medicine, University of Florence, Florence 50134, Italy

Vincenzo Di Pilato, Department of Surgical Sciences and Integrated Diagnostics, University of Genoa, Genoa 16126, Italy

Matteo Ramazzotti, Marco Pallecchi, Department of Biomedical, Experimental and Clinical "Mario Serio", University of Florence, Florence 50134, Italy

Gian Maria Rossolini, Microbiology and Virology Unit, Florence Careggi University Hospital, University of Florence, Florence 50134, Italy

Gianluca Bartolucci, Department of Neurosciences, Psychology, Drug Research and Child Health, University of Florence, Florence 50019, Italy

Corresponding author: Amedeo Amedei, BSc, PhD, Professor, Reader (Associate Professor), Department of Clinical and Experimental Medicine, University of Florence, Viale Pieraccini, 6, Florence 50139, Italy. aamedei@unifi.it

Abstract

BACKGROUND

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by persistent systemic inflammation and immune activation, even in patients receiving effective antiretroviral therapy (ART). Converging data from many cross-sectional studies suggest that gut microbiota (GM) changes can occur throughout including human immunodeficiency virus (HIV) infection, treated by ART; however, the results are contrasting. For the first time, we compared the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile of treatment-naïve patients before starting ART and after reaching virological suppression, after 24 wk of ART therapy. In addition, we compared the microbiota composition, microbial metabolites, and cytokine profile of patients with CD4/CD8 ratio < 1 (immunological non-responders [INRs]) and

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CD4/CD8 > 1 (immunological responders [IRs]), after 24 wk of ART therapy.

AIM

To compare for the first time the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile of treatment-naïve patients before starting ART and after reaching virological suppression (HIV RNA < 50 copies/mL) after 24 wk of ART.

METHODS

We enrolled 12 treatment-naïve HIV-infected patients receiving ART (mainly based on integrase inhibitors). Fecal microbiota composition was assessed through next generation sequencing. In addition, a comprehensive analysis of a blood broad-spectrum cytokine panel was performed through a multiplex approach. At the same time, serum free fatty acid (FFA) and fecal short chain fatty acid levels were obtained through gas chromatography-mass spectrometry.

RESULTS

We first compared microbiota signatures, FFA levels, and cytokine profile before starting ART and after reaching virological suppression. Modest alterations were observed in microbiota composition, in particular in the viral suppression condition, we detected an increase of *Ruminococcus* and *Succinivibrio* and a decrease of *Intestinibacter*. Moreover, in the same condition, we also observed augmented levels of serum propionic and butyric acids. Contemporarily, a reduction of serum IP-10 and an increase of IL-8 levels were detected in the viral suppression condition. In addition, the same components were compared between IRs and INRs. Concerning the microflora population, we detected a reduction of *Faecalibacterium* and an increase of *Alistipes* in INRs. Simultaneously, fecal isobutyric, isovaleric, and 2-methylbutyric acids were also increased in INRs.

CONCLUSION

Our results provided an additional perspective about the impact of HIV infection, ART, and immune recovery on the "microbiome-immunity axis" at the metabolism level. These factors can act as indicators of the active processes occurring in the gastrointestinal tract. Individuals with HIV-1 infection, before ART and after reaching virological suppression with 24 wk of ART, displayed a microbiota with unchanged overall bacterial diversity; moreover, their systemic inflammatory status seems not to be completely restored. In addition, we confirmed the role of the GM metabolites in immune reconstitution.

Key Words: HIV; Antiretroviral therapy; Microbiome-immunity axis; Microbiota; Cytokines; Short chain fatty acid; Inflammation; Immunological responders; Viremia

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Core Tip: Even in patients receiving effective antiretroviral therapy (ART), human immunodeficiency virus type 1 infection is characterized by persistent systemic inflammation and immune activation. Changes in the gut microbiota can occur with including human immunodeficiency virus infection and treatment with ART; however, the data are still conflicting. For these reasons, we compared the fecal microbial composition and serum cytokine profile of treatment-naïve patients before starting ART and after virological suppression. Finally, we evaluated the microbiota composition, microbial metabolites, and cytokine profile of patients with CD4/CD8 ratio < 1 and CD4/CD8 > 1 (immunological responders).

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INTRODUCTION

The mutual interaction between the human microbiota and the immune system defines the so-called "microbiome-immune axis". This axis has also been associated with several diseases, including human immunodeficiency virus (HIV) infection[1]. Indeed, a key place for HIV replication is the gastrointestinal tract. HIV replication in the gastrointestinal tract results in a severe depletion of $CD4^+$ T cells that leads to decreased function of the epithelial barrier, allowing microbes and microbial products to be translocated, which contributes to the chronic inflammatory response[2]. HIV replication can also result in a microbial dysbiosis condition[3-5], which has been correlated with increases in markers of disease progression, immune activation, and microbial translocation[3,5-7]. Notably, HIV-infected people harbour a distinct gut microbiota (GM)[8,9] with a *Prevotella*-rich community composition, typically observed in individuals from agrarian cultures or with carbohydrate-rich, protein- and fat-poor diets[10]. In addition, the significant subversion of the *Bacteroidetes* and *Proteobacteria* phyla, with an imbalanced *Prevotella/Bacteroides* species ratio and an abundance in *Enterobacteriaceae,* is one of the most persistent changes documented in untreated HIV infection[11-13]. Moreover, the increased number of gut-resident bacteria capable of directly producing inflammation can be a probable mechanistic link between HIV-associated dysbiosis and high systemic immune activation[14]. However, converging data from cross-sectional studies suggest that the GM composition and its related immune response can change over the progression of HIV infection. In particular, correlating the composition of the gastrointestinal tract microbiome to immune activation, circulating bacterial products and clinical parameters, a decrease of commensal species, and a gain of pathogenic taxa was observed in HIV+ subjects compared to controls[15]. Additionally, analysing the functional gene content of the GM in HIV+ patients and the metabolic pathways of the bacterial community associated with immune dysfunction, the metagenome sequencing revealed an altered functional profile with significant interactions between the bacterial community, their altered metabolic pathways, and systemic markers of immune dysfunction[16]. Furthermore, analysing the associations between the innate lymphoid cell (ILC) cytokines and measures of virologic, immunologic, and microbiome indices, it was observed that inflammatory ILCs contribute to gut mucosal inflammation and epithelial barrier breakdown, important features of HIV-1 mucosal pathogenesis[17]. Despite growing evidence that the GM has a role in HIV pathogenesis[11,18-20], the results were contrasting, with some studies suggesting an influence and others no HIV influence on microbial diversity[1,21] and composition[22,23]. However, many studies on the GM in HIV-infected patients are often carried out with a lack of adjustment for confounding factors, such as diet and use of drugs[24,25].

Currently, antiretroviral therapy (ART) has increased the life expectancy of HIV-infected patients, approximating it to that of the general population[26]. Interestingly, chronic inflammation and GM alterations persist in patients virologically suppressed by ART[27]. These data implicate that re-shaping the microbiota may be an adjuvant therapy in patients commencing successful ART[28]. On the other hand, suppressive ART appears to have a limited effect on the restoration of the GM[13,25,29,30]. Although the gut microbial composition of ART-treated people differs from that of untreated people, the former also have a different microbial community structure compared to the HIV-uninfected population[31,32]. These findings raise the possibility that persistent gut dysbiosis may play a role in the development of residual clinical illness after ART.

Currently, the CD4/CD8 ratio is considered one of the best-used markers of immune reconstitution. Notably, a low CD4/CD8 ratio is associated with an increased risk of non-AIDS-related diseases[33]. Furthermore, the differences between the elements of the microbiome-immune axis between patients with normalized or non-normalized CD4/CD8 ratio during ART have not been elucidated so far[34,35]; however, this question is recognized as a current research gap.

Moreover, with a better understanding of the microbiota-immune axis, it is now known that in addition to the intestinal flora itself, its metabolites are also involved in regulating vital host activities, such as energy metabolism, cell-to-cell communication, and host immunity. Short-chain fatty acids (SCFAs) are important metabolites able to modulate the production of immune mediators, such as key cytokines for the repair

and maintenance of epithelium integrity[36]. In addition, the SCFAs modulate the activity of T cells and decrease the overexpression of histone deacetylase, particularly butyric and valeric acids[37]. SCFAs are an important link between microflora and the immune system; they involve different molecular mechanisms and cellular targets, are essential for the maintenance of intestinal homeostasis, and finally play a role in HIV infection[38].

The purpose of this prospective observational study was to compare for the first time the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile of treatment-naïve patients before starting ART and after reaching virological suppression (HIV RNA < 50 copies/mL) after 24 wk of ART. An additional aim was to correlate the GM composition, microbial metabolites, and cytokine profile of patients with CD4/CD8 ratio < 1 and CD4/CD8 > 1 after antiretroviral therapy.

MATERIALS AND METHODS

Patients

The study population, composed of 12 treatment-naïve HIV-infected patients receiving ART mainly based on integrase inhibitors, was enrolled between April 2018 and May 2019 at the Department of Infective and Tropical Disease at University Hospital of Careggi, Florence, Italy (Table 1). The study was approved by local institutional review boards and written informed consent was obtained from patients before participation (Rif CEAVC 15035).

We conducted a prospective observational cohort study comparing the changes occurring in the fecal microbiota, serum and fecal SCFA, serum free fatty acids (FFAs), and serum cytokines of patients with HIV-1 infection before ART (T0) and after 24 wk (T1). In addition, patients were divided into two groups according to whether they were immunological responders (IRs, n = 6) or not (INRs, n = 6) (INRs and IRs, based on the normalization of CD4/CD8 ratio: < 1 or ≥ 1 after 24 wk of ART, respectively). Patients who had used antibiotics, probiotics, or prebiotics or had experienced diarrhoea or digestive symptoms within the previous 1 mo were excluded.

Personal data, ART regimen, HIV-RNA values, and number of $CD4^+$ and $CD8^+$ T cells prior to ART starting and at the time of virologic suppression were included in the analysis (Table 1). In this pilot exploratory study, no formal sample size calculation was performed. All patients followed a Mediterranean diet.

Plasma HIV-RNA was measured using Test v1.5 Roche COBAS AmpliPrep, Roche TaqMan HIV-1 Test v2.0 (Roche Diagnostics, Branchburg, NJ, United States) and Siemens Versant K PCR (Siemens Healthcare GmbH, Erlangen, Germany), with lower limits of detection of 50, 20, and 37 copies/mL, respectively.

The T cell counts of patients were determined using a FACScanto flow cytometer (BD Immunocytometry Systems)[10]. Immunophenotyping of peripheral blood lymphocytes was analysed by three-color flow cytometry (Epics XL Flow Cytometry System; Beckman Coulter, United States) as previously described[39]. Freshly collected EDTA anticoagulated whole blood was incubated and tested with a panel of monoclonal antibodies directed against fluorescein isothiocyanate/phycoerythrin/peridinin chlorophyll protein combinations of CD3/CD4/CD8, CD3/CD16CD56/CD19, HLA-DR/CD8/CD38, and CD4/CD8/CD28 and isotype controls (Immunotech, France).

At each time point (0 and 24 wk after study enrolment), we collected blood and fecal samples. After collection, stool samples were immediately frozen and stored at −80 °C until DNA extraction. Fecal samples were used to assess the microbiota composition and SCFAs, and while blood samples were used to measure SCFAs and FFAs and a panel of 27 selected cytokines.

Study follow-up

Patients underwent medical visits at 0 and 24 wk after study enrolment. They also underwent a comprehensive physical examination and medical history inquiry, urine toxicology panel testing, clinical laboratory tests including plasma HIV RNA, specimen collection, and detailed behavioural questionnaire survey. Demographic and clinical data were collected in a specific questionnaire and reported in an appropriate database, including the time point of follow-up in months; the participant's gender, age, weight, and height; $CD4^+$ and $CD8^+$ T cell counts; the CD4/CD8 ratio; HIV-1 RNA levels, ART, and antibiotic use. If subjects had to start antibiotics, they provided a last fecal sample and the study follow-up was immediately terminated.

Table 1 Features of the enrolled patients

	Age	Sex	ART regimen	Comorbidities	Timepoints (wk)	Viral load (copies/mL)	CD4+ cells/mm^3	CD8+ cells/mm^3	CD4/CD8 ratio
1	37	Male	3TC/ABC/DTG	No	T0	597463	110	420	0.3
					T24	< 20	520	832	0.6
2	38	Male	FTC/TDF/EVG/C	No	T0	4489	630	670	0.9
					T24	TND	831	740	1.1
3	34	Male	FTC/TDF/EVG/C	No	T0	165516	253	725	0.3
					T24	TND	504	363	1.4
4	39	Male	FTC/TDF/EVG/c	No	T0	859883	360	974	0.4
					T24	33	781	986	0.8
5	38	Male	3TC/ABC/DTG	No	T0	4860	1341	928	1.4
					T24	TND	1881	988	1.9
6	41	Male	FTC/TDF/RPV	Atrial fibrillation	T0	213	814	690	1.2
					T24	TND	845	519	1.6
7	25	Male	3TC/ABC/DTG	No	T0	23098	516	1149	0.4
					T24	< 20	942	1019	0.9
8	22	Male	FTC/TAF/EVG/c	No	T0	12188	654	1055	0.6
					T24	TND	668	733	0.9
9	48	Male	3TC/ABC/DTG	No	T0	175	833	1520	0.5
					T24	TND	941	1258	0.7
10	53	Male	3TC/ABC/DTG	Hypertension, HCV	T0	40545	863	1196	0.7
					T24	TND	612	515	1.2
11	40	Male	3TC/ABC/DTG	No	T0	859000	399	980	0.4
					T24	39	648	652	1
12	51	Male	FTC/TDF DTG	Diabetes	T0	4410	884	1066	0.8
					T24	< 20	1130	1261	0.9

ART: Antiretroviral therapy; 3TC: Lamivudine; ABC: Abacavir; DTG: Dolutegravir; FTC: Emtricitabine; TDF: Tenovir; EVG/c: Elvitegravir/cobi; RPV: Rilpivirine.

Fecal microbiota characterization

Total genomic DNA was extracted from frozen (-80 °C) stool samples, collected at different time points (weeks 0 and 24; T0 and T24), using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of purified DNA were assessed using the NanoDrop ND-1000 (Thermo Fisher Scientific, WalthAP, US) and the Qubit Fluorometer (Thermo Fisher Scientific), respectively.

Extracted DNA samples were sent to IGA Technology Services (Udine, Italy) where amplicons of the variable V3–V4 region of the bacterial 16S rRNA gene were sequenced (2 × 300 bp paired-end) on the Illumina MiSeq platform, according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol[40].

Sequencing results were analysed using the QIIME 2 suite (Quantitative Insights Into Microbial Ecology)[41]. Briefly, following raw reads denoising (*i.e.*, estimation of error rates, removal of chimeric and singleton sequences, and join of denoised paired-end reads) using DADA2 (Divisive Amplicon Denoising Algorithm 2)[42], denoised reads were dereplicated and amplicon sequence variants (ASVs) were inferred. Taxonomic classification of inferred ASVs was performed using a Naive Bayes classifier trained on the SILVA 16S reference database (release 132) (https://www.arb-silva.de/documentation/release-132/).

Evaluation of fecal short chain fatty acids and serum free fatty acids by gas chromatography-mass spectrometry

The fecal SCFAs, in particular acetic, propionic, butyric, isobutyric, isovaleric, 2-methylbutyric, valeric, and hexanoic acids, were analyzed using an Agilent GC-MS system composed with a 5971 single quadrupole mass spectrometer, a 5890 gas-chromatograph, and a 7673 auto sampler. The chemicals, GC-MS conditions, and calibrations parameters are reported in supporting information (Tables S1-S4)[43]. Fecal samples were collected in 15-mL Falcon tubes and stored at -80 °C. Just before the analysis, each sample was thawed, weighted (between 0.5-1.0 g), and added to sodium bicarbonate 10 mmol/L solution (1:1 w/v) in a 1.5 mL centrifuge tube. The obtained suspension was briefly stirred in a vortex apparatus, extracted in an ultrasonic bath (for 5 min), and then centrifuged at 5000 rpm (for 10 min). The supernatant was collected and transferred into a 1.5 mL centrifuge tube (sample solution). The SCFAs were finally extracted as follows: An aliquot of 100 μL of sample solution was added to 50 μL of internal standard mixture, 1 mL of tert-butyl methyl ether, and 50 μL of 1.0 mol/L HCl solution in a 1.5 mL centrifuge tube. Afterwards, each tube was shaken in a vortex apparatus for 2 min and centrifuged at 10000 rpm for 5 min, and finally the solvent layer was transferred into an autosampler vial and analyzed by the GC-MS method. Each sample was prepared and processed, by the method described above, three times. In addition, serum FFAs, classified as SCFAs (acetic, propionic, butyric, isobutyric isovaleric, 2-methylbutyri, and valeric acids), medium chain fatty acids (MCFAs; hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids), and long chain fatty acids (LCFAs; tetradecanoic, hexadecanoic, and octadecanoic acids) were analyzed with our previous described GC-MS protocol[44]. The chemicals, GC-MS conditions, GC-MS method, and calibrations parameters are reported in supporting information (Tables S5-S7).

Just before the analysis, each sample was thawed. The FFAs were extracted as follows: An aliquot of 300 μL of plasma sample was added to 10 μL of internal standard mixture, 100 μL of tert-butyl methyl ether, and 20 μL of 6 M HCl plus 0.5 mol/L NaCl solution in a 0.5 mL centrifuge tube. Afterwards, each tube was stirred in vortex for 2 min and centrifuged at 10000 rpm for 5 min, and finally the solvent layer was transferred into a vial with a microvolume insert and analyzed.

Molecular inflammatory response in serum

The inflammatory response in serum samples of patients and healthy controls was evaluated using a specifically assembled kit ProCartaPlex MixMatch Human 27 Panel for Luminex MAGPIX detection system (Affymetrix, eBioscience) following the manufacturers' instructions.

In detail, the panel included macrophage inflammatory protein-1α (MIP-1α), interleukin (IL)-27, IL-1β, IL-2, IL-4, IL-5, interferon gamma-induced protein 10 (IP-10), IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, interferon (IFN)-γ, IFN-α, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein 1(MCP-1), IL-9, P-selectin, IL-1α, IL-23, IL-18, IL-21, soluble intercellular adhesion molecule-1 (sICAM-1), IL-22, and E-selectin.

All measurements were performed in a blinded manner by a laboratory technician who was experienced in executing the technique. The levels of cytokines were estimated using a 5-parameter polynomial curve (ProcartaPlex Analyst 1.0). A value under the low limit of quantification (LLOQ) was considered as 0 pg/mL.

Statistical analysis

Statistical analyses on ASVs representing the bacterial community were performed in R (R Core Team, 2014) with the help of the packages phyloseq 1.26.1[45] and DESeq2 1.22.2[46], and other packages satisfying their dependencies, in particular vegan 2.5-5 [47]. Rarefaction analysis on ASVs was performed using the function rarecurve (step 50 reads), and further processed to highlight saturated samples (arbitrarily defined as saturated samples with a final slope in the rarefaction curve with an increment in ASV number per reads < 1e-5). For the cluster analysis (complete clustering on euclidean distance) of the entire community, the OTU table was first normalized using the total ASV counts of each sample and then adjusted using square root transformation. The coverage was calculated by Good's estimator using the formula $(1 - n/N) \times 100$, where n is the number of sequences found once in a sample (singletons), and N is the total number of sequences in that sample.

Richness, Shannon, Chao 1, and evenness indices were used to estimate bacterial diversity in each sample using the function estimate_richness from phyloseq[45]. The evenness index was calculated using the formula E = S/Log(R), where S is the

Shannon diversity index and R is the number of ASVs in the sample. Differences in all indices were tested using a paired Wilcoxon signed-rank test. The differential analysis of abundance at the ASVs as well as at the different taxonomic ranks (created using the tax_glom function in phyloseq) was performed with DESeq2[46] using a two group blocked by patient design in order to perform a paired test[48].

In addition, the software GraphPad Prism (v. 5) and Statgraphics Centurion XVI software were used for immunological data analysis. Numerical data are presented as the mean ± SD. The concentrations of several cytokines in some of the samples lay below the curve fit of the standards. To avoid the bias that would have been introduced by excluding these data, the concentrations of the implicated cytokine were set at half of the lower cut off of the test system, which was usually about 1 pg/mL. Outliers at the other end of the spectrum (higher than the mean ± SD) were identified *via* boxplots and were excluded from the statistical analysis. The comparisons between dependent groups were evaluated by the Wilcoxon matched pairs test, while the comparisons between the independent groups were assessed by the Mann-Whitney test. A P value less than 0.05 were considered statistically significant.

Data availability statement

The 16S rRNA sequence dataset has been deposited in the NCBI Sequence Read Archive (SRA) database and is available under the BioProject accession number PRJNA731648.

RESULTS

Comparison of fecal microbiota and metabolic and inflammatory profiles after ART

Modest differences in specific fecal microbiota taxa associated with HIV viremia: In the first part of our study, we compared the fecal microbiota and metabolic and inflammatory profile before and after ART starting, in order to examine potential changes resulting from HIV infection and ART therapy. We first analysed the longitudinal variation of fecal microbiota population in the same patients at T0 (HIV+ viremia - RNA > 50 copies/mL), defined as "high viremia" condition, and T24 (HIV+ suppression - RNA ≤ 50 copies/mL), defined as "viral suppression" condition. The alpha diversity of samples did not display significant differences for Chao, Shannon, and evenness indices (Figure 1). The analysis of the taxonomic composition revealed that more than 99% of the sequences collected were classified into four phyla: *Firmicutes* (65.46%), *Bacteroidetes* (21.54%), *Actinobacteria* (9.40%), and *Proteobacteria* (2.72%). In order to investigate similarity of patients' microbiota abundance profiles and to study the paired nature of sampling (*i.e.*, high viremia condition *vs* viral suppression condition), a cluster analysis and PCoA on normalized ASV counts were performed.

The hierarchical clustering evidenced that microbiota was not sufficiently altered after treatment (24 wk) to break individual compositions apart, resulting in a perfect matching of the two time points from the same patient (Figure 2A). This result was also confirmed by the PCoA (Figure 2B), which showed a substantial proximity of each patient at T0 and T24, indicating that, overall, the abundance profile of the single patient was not affected by the 24-wk therapy.

On the other hand, the paired comparison of the abundance of single microbial ranks revealed some significant (adj. $P < 0.05$, abs (logFC) ≥ 1) differences between the two samples groups. In particular, the genera *Ruminococcus 2* and *Succinivibrio* were found to be significantly increased in higher viral suppression condition. On the contrary, viral suppression was related with a decrease in the *Intestinibacter* genus (median abundance, ~1%) (Figure 3).

Analysis of fecal SCFAs displays no different layout between "high viremia" and "viral suppression" conditions: As we noticed minor changes in fecal microbiome profile (just at the order and genus levels), we wondered if the GM metabolic activity had been altered as well, and whether this activity might be masked by simply examining the microbiota composition. In order to evaluate the presence of alterations in GM metabolic activity, the levels of microbial linear and branched SCFAs were measured in fecal samples for each patient. However, the analysis of linear SCFA (acetic, propionic, butyric, and valeric acids), and branched SCFA (isobutyric, isovaleric, and 2-metilbutyric acids) abundance did not reveal any significant change after 24 wk of therapy for each patient.

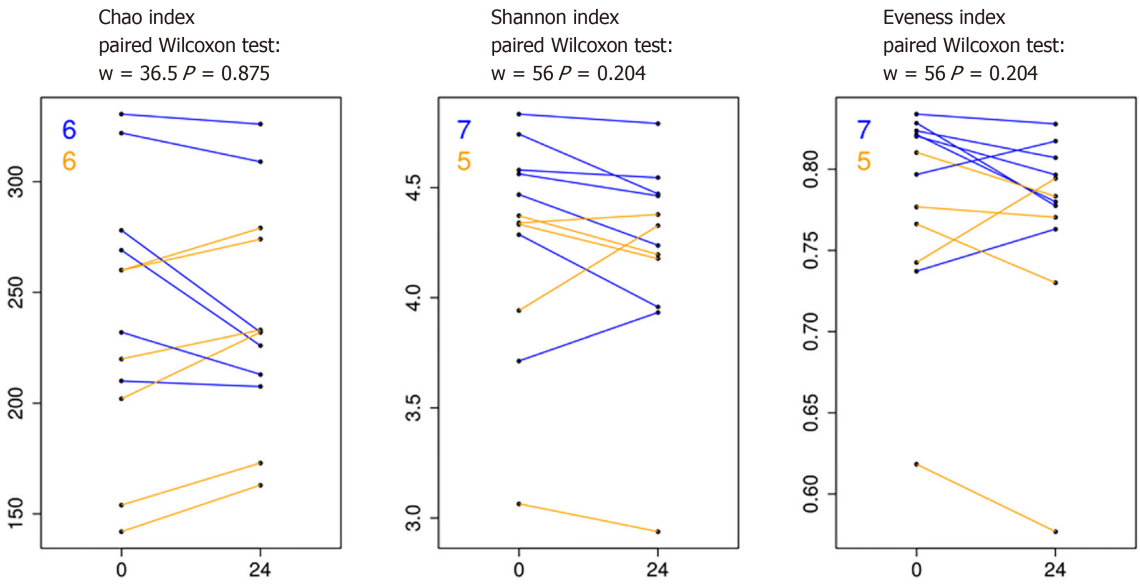


Figure 1 Box-plots showing alpha diversity indices (Chao1, Shannon, and evenness indices) in samples. Statistical differences were evaluated using paired Wilcoxon signed-rank test for Chao, Shannon, and evenness indices. *P* value less than 0.05 were considered statistically significant.

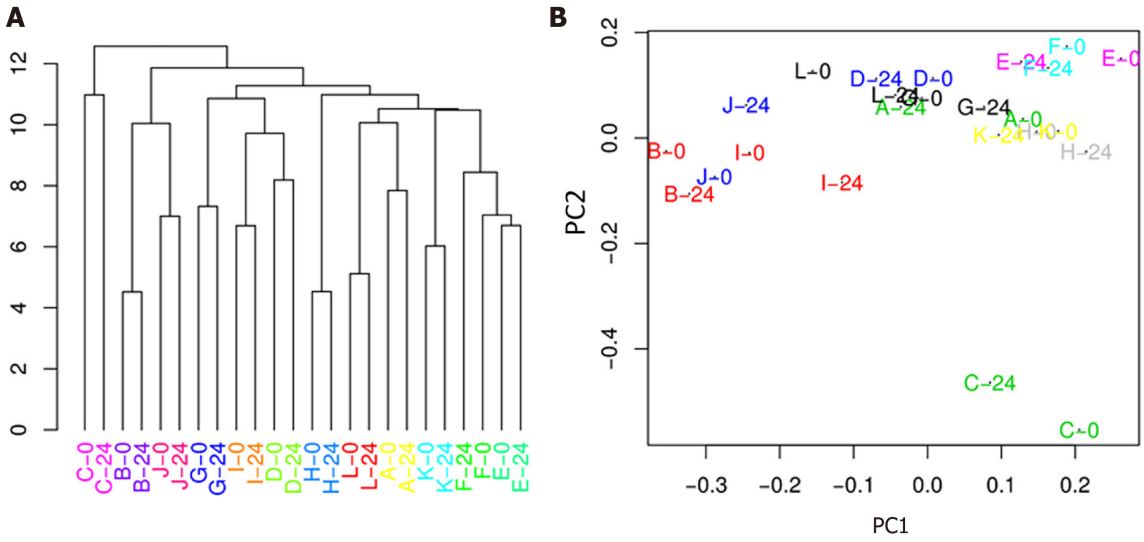


Figure 2 Cluster analysis (A) and principal coordinate analysis showing that samples do not separate into two groups depending on their condition (0-24 wk) (B).

Analysis of serum FFAs reveals a significantly different subgroup of SCFAs between "high viremia" and "viral suppression" conditions: As we did not report alterations in the composition of fecal SCFAs, we wanted to observe if there were any other alterations in metabolic output, by analyzing both microbial and host derived FFAs in serum. As known, the impairment of gut integrity due to dysbiosis condition, leads to translocation of microbial elements from the intestinal mucosa to the bloodstream, which is considered a major driving force of chronic immune activation [49] even in patients successfully treated with ART and achieving stable virological suppression[2].

The analysis of serum FFA levels showed a significant change of two SCFAs at T24 compared to the baseline. In particular, propionic and butyric acids were increased in viral suppression condition (Figure 4).

Inflammatory profile between high viremia and viral suppression conditions: As known, gut microbial dysbiosis is linked to aberrant immune responses, as alterations in the GM may induce the interruption of gut epithelial barrier integrity with subsequent microbial translocation, increased inflammation, and immune activation,

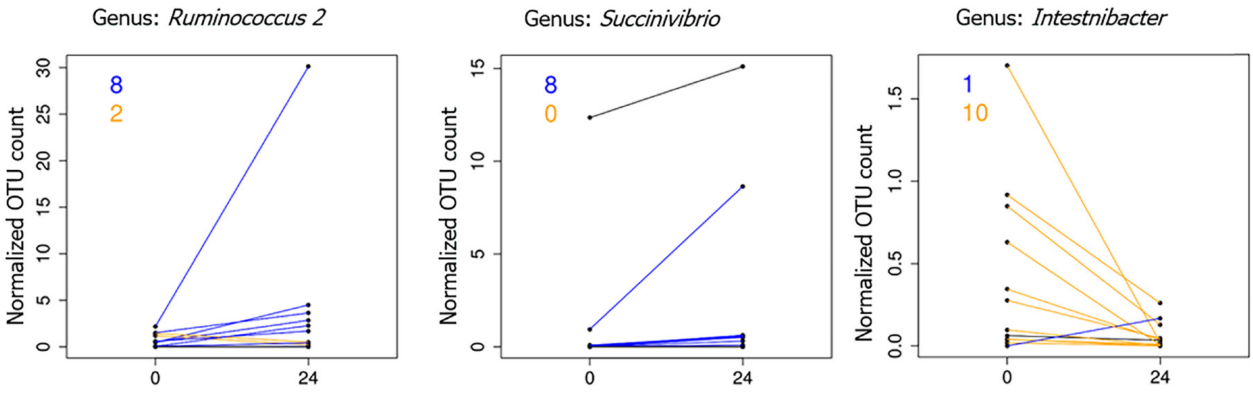


Figure 3 Segment plots depicting taxa with significantly differences between high viremia (time point 0) and viral suppression (time point 24) conditions. Lines connect paired samples and highlight the differences in normalized abundance for the indicated rank. Orange or blue colors highlight decrease or increase, respectively. Numbers in the top-left corner represent counts of increased (orange) and decreased (blue) measurement for paired samples.

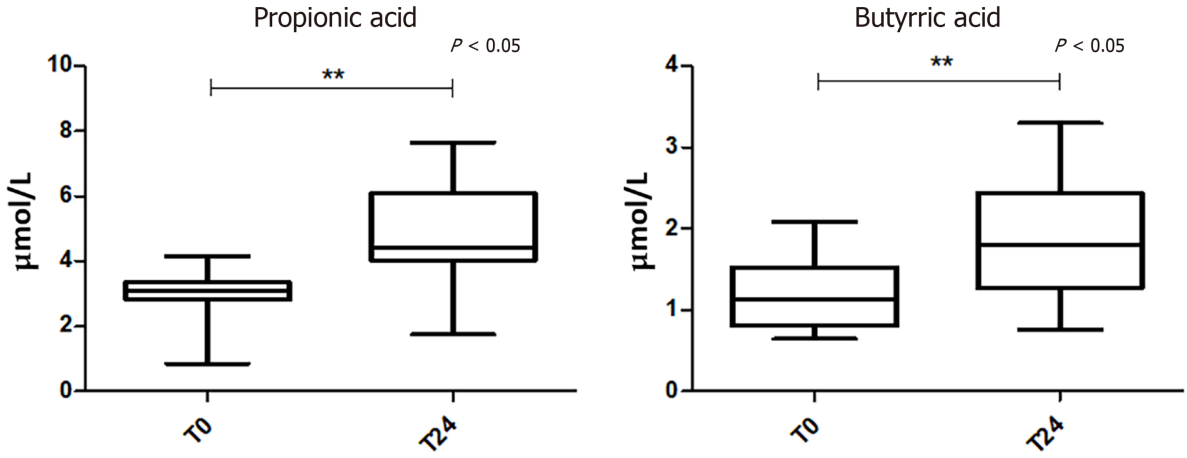


Figure 4 Boxplots showing statistically different levels of serum short-chain fatty acids between high viremia and viral suppressor patients, assessed by the Wilcoxon test. *P* value < 0.05 was considered statistically significant.

which are often accompanied by abnormal differentiation of immunological cells[6, 50]. Since we detected significant variations of microbial communities between high viremia and viral suppression conditions, we decided to characterize also the serum immunological profile by evaluating a panel of 27 cytokines between the two mentioned conditions. Among the 27 cytokines examined, we detected a significant reduction of IP-10 ($P = 0.0244$) and a significant increment of IL-8 levels ($P = 0.0547$) in the high viremia setting (Figure 5).

Association of GM composition and metabolic and inflammatory profiles with CD4⁺ T-cell counts

Correlation between fecal microbiota and CD4/CD8 ratio: In the second part of our study, we divided our cohort of patients into two groups: Immunological responders (IRs) and immunological non-responders (INRs), based on the CD4/CD8 ratio > 1 or < 1. In this condition, the analysis of microbiota revealed that, considering only taxa with an overall abundance higher than 1%, members of the *Faecalibacteria* genus were significantly reduced (adj. $P < 0.05$, logFC = 1.32) while members of the *Alistipes* genus were significantly increased in responders (adj. $P < 0.05$, logFC = 2.5) (Figure 6).

Different branched SCFA profiles in serum and fecal samples between IRs and INRs: As we observed significant variations in the composition of the fecal microbiota between IRs and INRs, we assessed if there were any other alterations in the fecal and serum microbial metabolites as linear and branched SCFAs derived from bacterial metabolism. We documented significant changes in isobutyric ($P = 0.01$), isovaleric ($P = 0.04$), and 2-methylbutyric ($P = 0.04$) acids, which were increased in IR fecal samples

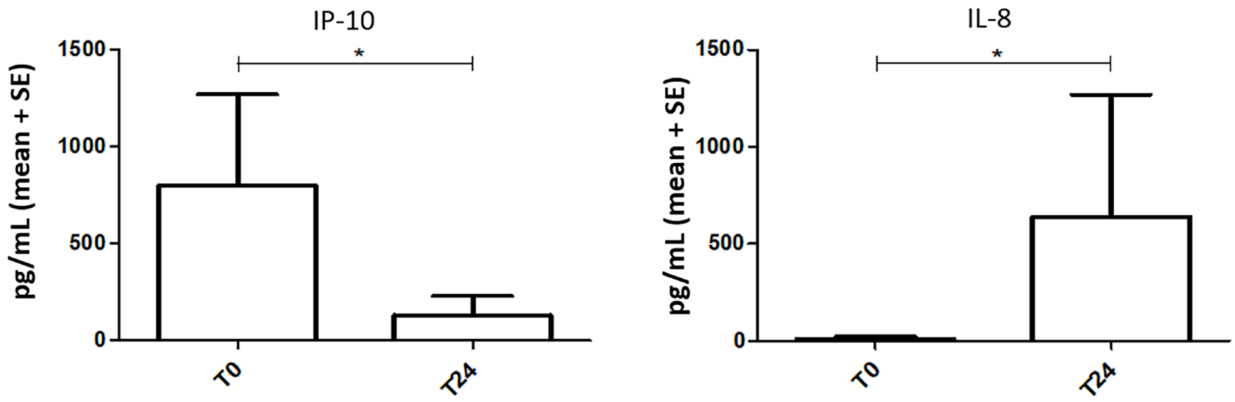


Figure 5 Boxplots showing statistically different levels of serum cytokines between high viremia and viral suppressor patients, assessed by the Wilcoxon test. A *P* value < 0.05 was considered statistically significant.

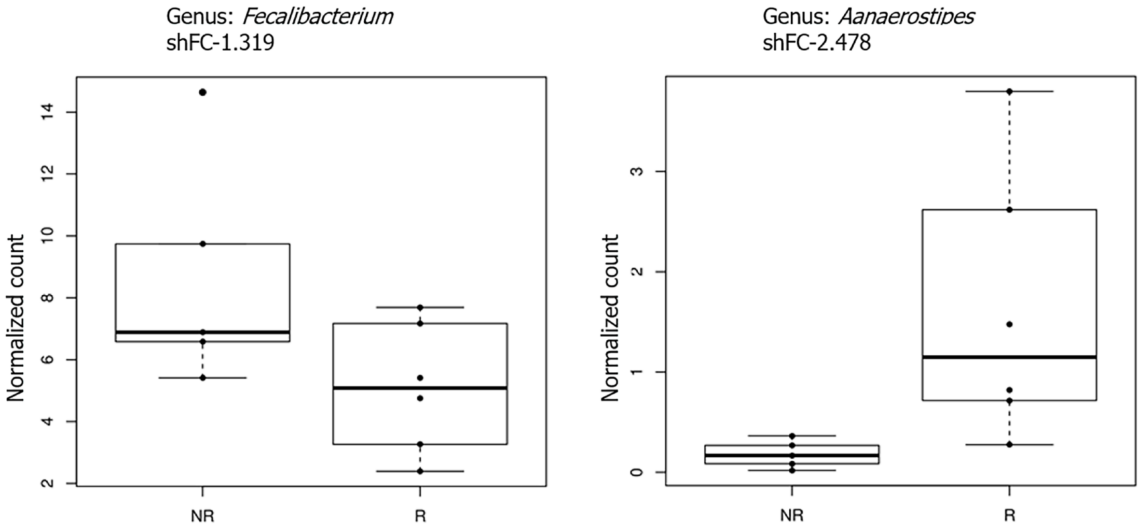


Figure 6 Boxplots showing the results of taxa-level differential abundance analysis between immunological responders and immunological non-responders at 24 wk. Plot titles report the shrunk Log2 fold change (according to the DESeq2 function lfcShrink). All results have a *P* value < 0.05. NR = INRs, R = IRs. IRs: Immunological responders; INRs: Immunological non-responders.

while we did not detect significant differences in serum samples (Figure 7).

Inflammatory profile shows no significant differences between IRs and INRs: Since we detected significant variations of microbial communities between IRs and INRs, we also evaluated the serum immunological profile. However, cytokine levels did not show significant variations between the IRs and INRs.

DISCUSSION

Currently, the mechanisms regulating the interplay between the host immune system and HIV-1, as well as the exact changes occurring in the GM composition and functionality, remain to be defined. To clarify the intricate relationships between the actors of the "microbiota-immunity" axis, we examined microbiota composition and functionality (SCFAs), serum inflammatory response, and FFA composition in individuals undergoing ART in different HIV infection settings.

Today, many studies on microbiota have been performed chiefly comparing HIV-infected and uninfected individuals, revealing a reduced GM diversity (the so-called HIV-associated dysbiosis) and an independent association between alpha-diversity of microbiota and peripheral levels of $CD4^+$ T cell count in treatment-naïve HIV-infected patients[28]. However, cross-sectional studies may not be suitable to provide information about cause-and-effect relationships, whereas longitudinal ones could be

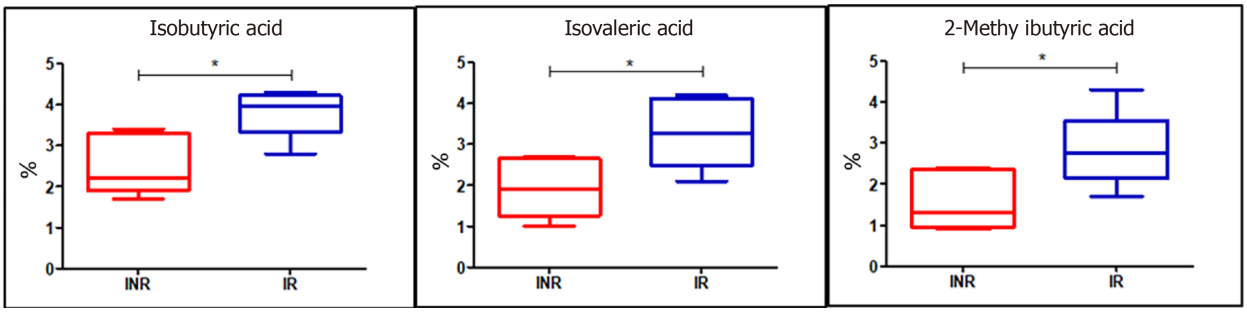


Figure 7 Boxplots showing statistically different fecal short-chain fatty acid abundances between immunological responders and immunological non-responders, assessed by the Mann-Whitney test. [a]*P* value < 0.05 was considered statistically significant.

more valid for examining such relationships. Besides, there is a lack of human longitudinal observations of the "microbiota-immunity" axis before and after first ART administration. Only in few longitudinal studies, where HIV-1-infected participants were followed after ART starting, data obtained on bacterial flora showed that shifts in the fecal microbiota persisted in a number of patients[10,28]. On the other hand, a recent study by Dillon *et al*[14] failed to find a significant change in a single time point study of the stool of HIV-1-infected patients.

In this study, we first performed a longitudinal investigation evaluating the GM before the treatment and after "viral suppression" (T24). According to the longitudinal study conducted by Dillon *et al*[14], our results showed modest changes in the GM composition after ART; indeed, we did not assess significant differences in phylum composition. However, the paired comparison of the abundance of single bacterial taxa revealed a significant alteration at the genus level between the two sample groups (Figure 3). In particular, the genera of *Ruminococcus*, and *Succinivibrio* were significantly increased after ART and the viral suppression. Conversely, the genus of *Intestinibacter* was significantly decreased in the same condition. We hypothesize that the slight change between the two groups may be due to persistent inflammation (related to microbial translocation and reduced immunoregulatory function), HIV latency throughout the gut, and direct effects of antiretroviral drugs on the bacterial population. Moreover, our results are in accordance with other longitudinal previous studies in non-human primates, which allowed to control for confounders affecting human studies[51,52]. We also reported an increase of the genus *Succinivibrio* (*Proteobacteria* phylum) between the two samples groups. In addition, in agreement with our data, the proportion of the rare genus *Succinivibrio*, was also found considerably high in the stool of Japanese patients treated with ART[53]. One of the possible reasons for the contradictory results reported in the examined different studies may include the cross-sectional nature of the study, the used sampling method (stool swab *vs* stool), and the microbial taxon level applied.

Based on our findings, the 24 wk of ART inhibited HIV-1 viral replication effectively (indeed, all enrolled patients reached viral suppression), but did not heavily affect the overall bacterial composition of the gut microenvironment. The modest GM diversity that we observed between the two sample groups might be associated with the lowering of viremia. However, there was evidence that ART also induces changes in the gut microbiome, unrelated to HIV infection. Some authors have implied that ART may enhance dysbiosis, which is consistent with the high frequency of gastrointestinal side effects of this treatment[28,54].

As the GM influences the immune system through their bacterial metabolites, like SCFAs[55,56], we measured SCFA levels in blood and stool samples, in order to have a more accurate assessment of microbial metabolism after the ART. As known, the main SCFAs include, in order of proportion, acetic, propionic, and butyric acids that are produced by fibres fermentation by gut bacteria, particularly by members of the *Firmicutes* phylum[57]. Interestingly, for the first time, we observed a significant change of two serum SCFAs after the ART. In particular, propionic and butyric acids were increased in "viral suppression" condition. This altered SCFA profile may indicate a potential role for the SCFA synthesis pathway in the regulation of the HIV "microbiota-immunity" axis during effective ART. Notably, we did not observe any significant SCFA changes in stool samples, probably because in the colon, about 95% of the produced SCFAs are rapidly absorbed by large intestinal mucosal cells while the remaining 5% are secreted in the feces[58]. Propionate is only present at a low concentration in the periphery because it is metabolized in the liver[59]. It has been shown

that butyrate may reduce gut inflammation by inducing the regulatory T cells (Tregs) and modulating activation of antigen-presenting cells[17]. We may speculate that bacterial flora responds reciprocally to inflammation by increasing the biosynthesis of anti-inflammatory and pro-solving lipid mediators that circulate in the bloodstream. Altogether, it is plausible that immune system-bacteria synergism mediates solutions to inflammation. On the contrary, as previously reported, some studies have found that butyrate-producing bacteria are selectively reduced in stool samples from HIV-infected compared to non-infected subjects[17,54]. In particular, Serrano-Villar *et al*[60] found that HIV-infected individuals had a distinct SCFA profile in stool compared to HIV-negative controls, with increased propionate and lower levels of acetate. No data from the literature are available regarding SCFA levels in HIV$^+$ serum samples, except a study of Segal *et al*[61] reporting that higher values of serum SCFAs, in consequence of an increased abundance of pulmonary anaerobic bacteria in HIV+ patients on ART, inhibited the immune response to *M. tuberculosis*, likely enhancing tuberculosis susceptibility. They observed that baseline serum butyrate and propionate were associated with the subsequent increasing hazard of tuberculosis. Moreover, we also evaluated serum FFA composition before and after ART treatment. Indeed, increased levels of FFA and proinflammatory cytokines have been reported in some HIV-infected patients under ART (reviewed in reference[62]). However, we did not appreciate any difference at the examined two time points.

Regarding the inflammation tone, there is consensus that a pro-inflammatory status remains active even after ART initiation in most patients[63,64]. Since the HIV life cycle is suppressed through ART in treated patients, the chronic inflammatory status observed in patients is maintained by factors secondary to HIV replication, including microbial translocation and reduced immunoregulatory function. In order to evaluate the inflammatory status after ART, we measured a panel of selected multifunctional effector molecules of the immune response in serum. Among the measured cytokines, we observed a decrease of IP-10 (P = 0.0244) after the treatment, confirming the downregulation of this chemokine production in patients with HIV infection during ART[65-69]. IP-10 is involved in trafficking immune cells to inflammatory sites, and it is considered an important pro-inflammatory factor in the HIV disease process. It has been observed that its levels can be reduced, but not to normal levels, by ART administration. Interestingly, IP-10 was consistently associated with HIV disease progression (based on CD4$^+$ counts) during the period[70], suggesting its potential for use as an indicator of HIV infection and/or a therapeutic target for HIV treatment[71]. On the other hand, in agreement with recent data, we observed a significant increased trend of IL-8 levels (P = 0.0547) with suppressed viral load after 24 wk of ART. Indeed, increased IL-8 levels were observed in HIV-infected individuals on ART[72]. It has been shown that during HIV-1 infection, IL-8 plays an important role in the recruitment of CD4$^+$ T cells to the lymph nodes, thus generating more targets for viral replication. Our results may suggest that increased IL-8 Levels may represent a hallmark of chronic inflammation in HIV$^+$ patients on ART. In accordance with our findings, Wada *et al*[73] observed significantly higher circulating IL-8 levels in HIV$^+$ men on ART with suppressed viral load in comparison to HIV-uninfected men.

It is now established that the gut microbiome may play a crucial role in the immune activation in HIV-infected patients treated with ART[5,64,73-75]. Recently, several studies have reported that GM is associated with CD4$^+$ T cell recovery in HIV-infected patients, playing an essential role in the reconstitution of immune function[76-78]. The potential mechanism includes the formation of a virus shelter, resistance to ART, promotion of intestinal mucosal barrier damage, and further entry of intestinal bacteria and their metabolites into the circulatory system, resulting in long-term immune activation, inflammation, and metabolic disorders such as cardiovascular diseases, diabetes mellitus, liver steatosis, and lastly, cancer[8]. Although it remains unclear whether an altered immunity after HIV infection drives dysbiosis or *vice versa*, the gut dysbiosis, immune dysfunction, epithelial damage, and microbial translocation are still evident even in the setting of ART-mediated viral suppression, which might be the treatment dilemma for HIV infection at present. Despite numerous studies of the microbiota in HIV-infected patients, there are relatively few reports discussing the compositional GM changes in patients with different immune responses to ART[79, 80].

To investigate the role of GM in immunomodulation and immune reconstitution and which bacterial metabolites are implicated, in the second part of the study, we divided the patients into two groups: Patients with CD4/CD4 ratio < 1 with insufficient reconstitution of CD4$^+$ T cells despite achieving virological suppression after 24 wk of ART and those with CD4/CD8 ≥ 1 who reached a robust reconstitution of CD4$^+$ T cells. We found that the *Anaerostipes* genus was significantly augmented in IRs; on

the contrary, the *Faecalibacterium* genus was significantly increased in INRs. Notably, *Faecalibacterium* has been reported as the anti-inflammatory commensal genus[81]. It has been positively correlated with the CD4/CD8 ratio and anti-correlated with inflammation markers and LPS in a recent study in HIV-infected patients[82].

Regarding microbial metabolites, we detected a significant increase in fecal isobutyric, isovaleric, and 2-methylbutyric acids in the IRs. However, we found that the changes associated with the IR group were not evident in the blood. Based on our results, we hypothesized that changes at the genus level in the gut ecosystem in HIV-infected patients undergoing ART might thus be both a consequence and a potential cause of the recovery of systemic immunity.

Our study had some limitations. First, a low number of patients were enrolled to investigate the elements of the microbiota-immunity axis and it cannot determine whether the altered GM contributed to or was caused by immune dysfunction. Second, only the effects of 24-wk ART were observed in our study, and to establish a more meaningful connection between GM and microbial/immune parameters, future studies should investigate the GM alterations and the restoration of immune function after long-term effective ART. Finally, the microbiota of feces was a proxy for GM in this study, which was the only realistic sample for a non-invasive study. However, fecal microbiota may only represent the GM composition in the lumen rather than on the mucosal surfaces, which is an important distinction because the mucosa-associated microbiota potentially interacts with the gut-associated lymphoid tissue in HIV-1-infected patients directly.

CONCLUSION

Our results provided an additional vision about the impact of HIV infection, ART, and immune recovery in the microbiota-immunity axis at the metabolism level, which are an indicator of the active processes occurring in the gastrointestinal tract. In summary, we demonstrated that patients infected by HIV-1, after reaching virological suppression with ART, displayed a fecal microbiota with unchanged overall bacterial diversity except for few genera. Although 24 wk of treatment with ART was effective, the systemic inflammatory tone was not completely restored despite the anti-inflammatory serum butyrate increment. In addition, we confirmed the role of the GM in immune reconstitution, with the possible implication of bacterial metabolites; however, changes in the gut ecosystem in HIV$^+$ patients undergoing 24 wk of ART may thus be both a consequence and a potential cause of the recovery of systemic immunity.

Future larger-scale, long-term ART and longitudinal studies that include functional metagenomic and metabolomic approaches to identify the roles of the specific differential phylotypes are required to better define the relationship between microbiota-immunity axis and HIV-1 infection and to provide new insights into the targeted treatment, improving the immune recovery and dampening inflammation.

ARTICLE HIGHLIGHTS

Research background

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by persistent systemic inflammation and immune activation, even in patients receiving effective antiretroviral therapy (ART). Converging data suggest that gut microbiota (GM) changes can occur throughout including human immunodeficiency virus (HIV) infection treated by ART.

Research motivation

ART has increased the life expectancy of HIV-infected patients; however, chronic inflammation and gut microbiota alterations persist in patients virologically suppressed by ART. These data suggest that re-shaping the microbiota may be an adjuvant therapy in patients commencing successful ART.

Research objectives

The purpose of this prospective observational study was to compare for the first time the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile of treatment-naïve patients before starting ART and after reaching

virological suppression (HIV RNA < 50 copies/mL) after 24 wk of ART.

Research methods

The authors enrolled 12 treatment-naïve HIV-infected patients receiving ART. Fecal microbiota composition was assessed through next generation sequencing, and a comprehensive analysis of a broad spectrum of cytokines in blood was performed through a multiplex approach. In addition, serum free fatty acid (FFA) and fecal short chain fatty acid (SCFA) levels were measured through GC-MS.

Research results

The authors compared microbiota signatures, FFA levels, and cytokine profile before starting ART and after reaching virological suppression. Modest alterations were observed on microbiota composition; moreover, in the same condition, we also observed augmented levels of serum propionic and butyric acids. A reduction of serum IP-10 and an increase of IL-8 level were detected in the viral suppression condition. Thereafter, the same components were compared between immunological responders and non-responders. Concerning the microflora population, we detected a reduction of *Faecalibacterium* and an increase of *Alistipes* in immunological non-responders. Simultaneously, fecal isobutyric, isovaleric, and 2-methylbutyric acids were also increased in immunological non-responders.

Research conclusions

The results provid an additional perspective about the impact of HIV infection, ART, and immune recovery on the "microbiome-immunity axis" at the metabolism level. These factors can act as indicators of the active processes occurring in the gastrointestinal tract.

Research perspectives

Future larger-scale, long-term ART and longitudinal studies that include functional metagenomic and metabolomic approaches to identify the roles of the specific differential phylotypes are required to better define the relationship between microbiota-immunity axis and HIV-1 infection and to provide new insights into the targeted treatment, improving the immune recovery and dampening inflammation.

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ORIGINAL ARTICLE

Case Control Study

Atrophic gastritis and gastric cancer tissue miRNome analysis reveals hsa-miR-129-1 and hsa-miR-196a as potential early diagnostic biomarkers

Greta Varkalaite, Evelina Vaitkeviciute, Ruta Inciuraite, Violeta Salteniene, Simonas Juzenas, Vytenis Petkevicius, Rita Gudaityte, Antanas Mickevicius, Alexander Link, Limas Kupcinskas, Marcis Leja, Juozas Kupcinskas, Jurgita Skieceviciene

ORCID number: Greta Varkalaite 0000-0003-3488-2171; Evelina Vaitkeviciute 0000-0003-2525-1597; Ruta Inciuraite 0000-0002-5806-6155; Violeta Salteniene 0000-0002-7881-945X; Simonas Juzenas 0000-0001-9293-0691; Vytenis Petkevicius 0000-0002-1964-5226; Rita Gudaityte 0000-0001-7489-8069; Antanas Mickevicius 0000-0002-6208-7039; Alexander Link 0000-0002-9514-4562; Limas Kupčinskas 0000-0002-8689-9023; Marcis Leja 0000-0002-0319-8855; Juozas Kupcinskas 0000-0002-8760-7416; Jurgita Skieceviciene 0000-0002-4893-6612.

Author contributions: Varkalaite G, Leja M, Kupcinskas L, Kupcinskas J and Skieceviciene J contributed to study conception and designed the research; Gudaityte R, Petkevicius V and Mickevicius A collected material and clinical data from the participants; Varkalaite G and Vaitkeviciute E performed all the investigations, analysis of data and drafted the manuscript; Juzenas S and Inciuraite R reviewed and edited the manuscript; Salteniene V revised the article for important intellectual content; Link A, Kupcinskas J and Skieceviciene J approved the final version of the manuscript.

Greta Varkalaite, Evelina Vaitkeviciute, Ruta Inciuraite, Violeta Salteniene, Simonas Juzenas, Limas Kupcinskas, Jurgita Skieceviciene, Institute for Digestive Research, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Vytenis Petkevicius, Juozas Kupcinskas, Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Rita Gudaityte, Antanas Mickevicius, Department of Surgery, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Alexander Link, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital, Magdeburg 39120, Germany

Marcis Leja, Institute of Clinical and Preventive Medicine & Faculty of Medicine, University of Latvia, Riga 1586, Latvia

Corresponding author: Jurgita Skieceviciene, PhD, Senior Researcher, Institute for Digestive Research, Lithuanian University of Health Sciences, A. Mickeviciaus street 9, Kaunas 44307, Lithuania. jurgita.skieceviciene@lsmuni.lt

Abstract

BACKGROUND

Gastric cancer (GC) is one of the most frequently diagnosed tumor globally. In most cases, GC develops in a stepwise manner from chronic gastritis or atrophic gastritis (AG) to cancer. One of the major issues in clinical settings of GC is diagnosis at advanced disease stages resulting in poor prognosis. MicroRNAs (miRNAs) are small noncoding molecules that play an essential role in a variety of fundamental biological processes. However, clinical potential of miRNA profiling in the gastric cancerogenesis, especially in premalignant GC cases, remains unclear.

AIM

To evaluate the AG and GC tissue miRNomes and identify specific miRNAs' potential for clinical applications (*e.g.*, non-invasive diagnostics).

Institutional review board statement: The study was approved by the Kaunas Regional Biomedical Research Ethics Committee.

Informed consent statement: All study participants provided informed consent prior to study enrollment.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at jurgita.skieceviciene@lsmuni.lt.

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METHODS

Study included a total of 125 subjects: Controls (CON), AG, and GC patients. All study subjects were recruited at the Departments of Surgery or Gastroenterology, Hospital of Lithuanian University of Health Sciences and divided into the profiling (n = 60) and validation (n = 65) cohorts. Total RNA isolated from tissue samples was used for preparation of small RNA sequencing libraries and profiled using next-generation sequencing (NGS). Based on NGS data, deregulated miRNAs hsa-miR-129-1-3p and hsa-miR-196a-5p were analyzed in plasma samples of independent cohort consisting of CON, AG, and GC patients. Expression level of hsa-miR-129-1-3p and hsa-miR-196a-5p was determined using the quantitative real-time polymerase chain reaction and $2^{-\Delta\Delta Ct}$ method.

RESULTS

Results of tissue analysis revealed 20 differentially expressed miRNAs in AG group compared to CON group, 129 deregulated miRNAs in GC compared to CON, and 99 altered miRNAs comparing GC and AG groups. Only 2 miRNAs (hsa-miR-129-1-3p and hsa-miR-196a-5p) were identified to be step-wise deregulated in healthy-premalignant-malignant sequence. Area under the curve (AUC)-receiver operating characteristic analysis revealed that expression level of hsa-miR-196a-5p is significant for discrimination of CON *vs* AG, CON *vs* GC and AG *vs* GC and resulted in AUCs: 88.0%, 93.1% and 66.3%, respectively. Comparing results in tissue and plasma samples, hsa-miR-129-1-3p was significantly down-regulated in GC compared to AG (P = 0.0021 and P = 0.024, tissue and plasma, respectively). Moreover, analysis revealed that hsa-miR-215-3p/5p and hsa-miR-934 were significantly deregulated in GC based on *Helicobacter pylori* (*H. pylori*) infection status [log2 fold change (FC) = -4.52, P-adjusted = 0.02; log2FC = -4.00, P-adjusted = 0.02; log2FC = 6.09, P-adjusted = 0.02, respectively].

CONCLUSION

Comprehensive miRNome study provides evidence for gradual deregulation of hsa-miR-196a-5p and hsa-miR-129-1-3p in gastric carcinogenesis and found hsa-miR-215-3p/5p and hsa-miR-934 to be significantly deregulated in *H. pylori* carrying GC patients.

Key Words: Gastric cancer; Atrophic gastritis; Tumorigenesis; *Helicobacter pylori*; MicroRNAs; Biomarkers

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Core Tip: In this research we aimed to evaluate microRNAs profiles of premalignant and malignant stages of gastric cancer (GC). To date this is the first study analyzing atrophic gastritis (AG) and GC tissue miRNomes in the subjects of European origin using next-generation sequencing approach. We showed that hsa-miR-196a-5p expression in tissue is significant for discrimination between controls and AG or GC, while hsa-miR-129-1-3p is potential candidate for non-invasive GC diagnostic. This study provides novel insights into complex GC pathogenesis cascade and might be highly significant for future studies of new AG or GC associated epigenetic markers or even diagnostic targets.

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INTRODUCTION

Gastric cancer (GC) is the one of the most common malignancy and the fourth leading cause of cancer-related death worldwide[1]. Studies show, that in most cases GC development is a stepwise process: Chronic gastric mucosa inflammation progresses to atrophic gastritis (AG) or intestinal metaplasia (IM), which eventually may become predisposition to GC. This complex cascade involves many factors: *Helicobacter pylori* (*H. pylori*) infection, lifestyle, dietary habits, and genetic or epigenetic alterations, including miRNA expression changes[2,3]. One of the major concerns in diagnostics of GC is poor survival rate and prognosis, while this tumor is usually diagnosed at late stages. Therefore, investigation of the molecular mechanisms that are critical in the complex GC pathological cascade may help to identify novel therapeutic targets and consequently improve the disease prognosis. MicroRNAs (miRNAs) are small (approx 22 nt) non-coding RNA molecules that regulate gene expression by binding to the specific sites within 3′ untranslated regions of target mRNAs[4,5]. MiRNAs play very important role in many physiological and pathological processes as well as tumorigenesis and may function as either tumor-suppressors or as oncogenic miRNAs[6-8]. Studies have reported numerous differentially expressed miRNAs in malignant gastric tissues including members of miR-20, miR-451, miR-148, miR-223 families[9-11]. Despite the previous efforts and conducted miRNA studies in GC, the miRNome characterization of premalignant gastric condition - AG - remains largely unknown.

In this study, we aimed to investigate miRNome profile through GC tumorigenesis cascade including precancerous lesions, such as AG. Also, expression of two miRNAs (hsa-miR-129-1 and hsa-miR-196a) was analyzed in plasma samples of the independent cohort of AG and GC patients. Tissue miRNome analysis results revealed distinct miRNA profiles comparing controls (CON), AG, and GC groups. Also, our study findings show that two miRNAs: Hsa-miR-129-1 and hsa-miR-196a may be a relevant biomarker for GC diagnostics.

MATERIALS AND METHODS

Study population

The study included a total of 125 CON and patients diagnosed with AG and GC, who were divided into the profiling cohort of 60 subjects and validation cohort of 65 subjects. Tissue samples of profiling cohort were collected during the years 2007-2015, while plasma of participants in validation cohort was collected from years 2011-2019 at the Departments of Surgery and Gastroenterology, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania). Clinical and phenotypic characteristics of subjects investigated in profiling and validation cohorts are presented in Table 1. *H. pylori* status was assessed using indirect ELISA to detect serum-specific IgG antigen (Virion/Serion GmbH, Germany). Control group consisted of subjects, who had no signs of atrophy or IM according to Operative Link on Gastritis Assessment (OLGA) staging system (stage 0)[12]. AG group consisted of individuals that had stage I-IV atrophy score in gastric mucosa by OLGA classification. Gastric adenocarcinoma in GC patients was verified by histology and classified according to the American Joint Committee on Cancer TNM Staging Classification and Lauren Classification[13,14]. Adjacent GC (GCaj) samples were biopsy samples obtained from endoscopically healthy appearing gastric mucosa at least 2 cm away from the primary tumor.

The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (approval No BE-2-10 and BE-2-31) and performed in accordance with the Declaration of Helsinki. All study participants provided written informed consent before enrollment.

Total RNA extraction

Total RNA, including small RNA fraction, was isolated from CON, AG and GC tissues using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantification of RNA was performed using Nanodrop2000 spectrophotometer (Thermo Fisher Scientific, United States) and quality of RNA samples was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, United States). Circulating nucleic acids, including circulating miRNA fraction, was isolated using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) according to manufacturer's instructions. All isolated samples were stored at -80 °C prior to further analysis.

Table 1 Demographic characteristics of profiling and validation cohorts

		Profiling cohort (*n* = 60)			Validation cohort (*n* = 65)		
		CON (*n* = 21)	AG (*n* = 19)	GC (*n* = 20)	CON (*n* = 11)	AG (*n* = 30)	GC (*n* = 24)
Age	Mean ± SD	58.29 ± 15.52	69.21 ± 8.78	64.95 ± 10.89	42.27 ± 12.89	68.01 ± 11.81	68.33 ± 11.27
Gender (*n*)	Male	5	3	15	5	9	18
	Female	16	16	5	6	21	6
***Helicobacter pylori* infection (*n*)**	Negative	12	10	8	-	17	9
	Positive	9	9	9	-	10	4
	Unknown	-	-	3	11	3	11
Differentiation grade (*n*)	G1	-	-	4	-	-	-
	G2	-	-	4	-	-	12
	G3	-	-	12	-	-	12
Lauren classification (*n*)	Diffuse	-	-	10	-	-	8
	Intestinal	-	-	10	-	-	13
	Mixed	-	-	-	-	-	2
	Unknown	-	-	-	-	-	1
T (*n*)	T1	-	-	6	-	-	3
	T2	-	-	2	-	-	5
	T3	-	-	8	-	-	9
	T4	-	-	4	-	-	6
	Unknown	-	-	-	-	-	1
N (*n*)	N0	-	-	10	-	-	6
	N1	-	-	2	-	-	5
	N2	-	-	3	-	-	4
	N3	-	-	5	-	-	8
	Unknown	-	-	-	-	-	1
M (*n*)	M0	-	-	7	-	-	14
	M1	-	-	2	-	-	9
	Unknown	-	-	11	-	-	1

SD: Standard deviation; CON: Control; AG: Atrophic gastritis; GC: Gastric cancer.

Small RNA-seq library preparation and next-generation sequencing

Small RNA libraries were prepared using Illumina TruSeq Small RNA Sample Preparation Kit (Illumina, United States) according to the manufacturer's protocol with 1 μg RNA input per sample followed by RNA 3′ adapter ligation, RNA 5′ adapter ligation, cDNA synthesis, polymerase chain reaction (PCR) amplification using unique barcode sequences for each sample and gel size-selection of small RNA library. The yield and quality of sequencing libraries were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, United States). The small RNA libraries were randomized, pooled 24 samples per lane and sequenced using Illumina HiSeq 2500 (1 × 50 bp single-end reads).

Bioinformatics analysis of small RNA-seq data

Analysis of raw small RNA-seq data was performed by nf-core/smrnaseq pipeline v.1.0.0 including Nextflow v.20.07.1[15], Java v.11.0.7, and Docker v.19.03.12. In brief, all steps consisted of read quality control using FastQC v.0.11.9, removing 3′ adapter sequences with TrimGalore! v.0.6.5, mapping to mature and hairpin miRNAs (miRBase v.22.1[16]), and GRCh37 human reference genome with Bowtie v.1.3.0[17].

After alignment and trimming sorted BAM files were used for further analysis with edgeR v.3.32.1[18] and mirtop v.0.4.23. MiRNA quality was assessed and summarized using MultiQC v.1.9[19]. Normalized counts were generated using isomiRs package and differential expression analysis was carried out using the DESeq2 Bioconductor package v.1.26.0[20]. The threshold for significant differential expression was Bonferroni[21] adjusted P-value < 0.05 and absolute value of log2 fold change (FC) |log2FC| > 1.

Validation of miRNA expression in plasma by reverse transcription quantitative real-time PCR

To validate differentially expressed miRNAs in plasma samples, isolated plasma circulating microRNA was reverse transcribed to cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, United States). The material was preamplified using the TaqMan PreAmp Master Mix (Applied Biosystems, United States) according to the manufacturer's protocol. Quantitative real-time PCR (RT-PCR) was performed using the TaqMan MicroRNA Assays: Hsa-miR-129* (Assay ID: 002298), hsa-miR-196a (Assay ID: 241070_mat) on 7500 Fast Real-Time PCR System (Applied Biosystems, United States). All RT-qPCR reactions were run in duplicate in a 20 μL reaction and the relative fold change in miRNA expression was estimated using the $2^{-\Delta\Delta Ct}$ method[22]. Ct values were normalized to the RNU6B (Assay ID: 001093, Thermo Fisher Scientific, United States) endogenous control.

Statistical analysis

Statistical analysis was performed using RStudio software (R v.3.6.3). Shapiro-Wilk normality test was used to test the normal distribution of data. For normally distributed data, statistical significance was assessed by Student's t-test. If the data did not pass normality tests was performed non-parametric Wilcoxon rank-sum test. A P < 0.05 was considered statistically significant. Area under the receiver operating characteristic curve (AUC-ROC) analysis was performed using pROC R package.

RESULTS

Small RNA sequencing reveals distinct miRNomes of healthy, premalignant, and malignant stages of GC

Small RNA sequencing of CON, AG, and paired GC (cancerous and adjacent) tissues in total identified 1037 miRNAs annotated in the miRBase v22.1. Sequencing yielded approx 250 M raw sequencing reads (from 359 K to 16 M reads per sample). After quality control steps 396 low-abundant and non-variable miRNAs and 5 outlying samples were removed resulting in 641 miRNAs and 75 samples which were used for further analysis (Supplementary Figures 1 and 2). The number of deregulated miRNAs corresponded to pathological cascade of GC development. The highest number of deregulated miRNAs were determined when comparing GC and CON groups (129 differentially expressed miRNAs, 82 up-regulated and 47 down-regulated; Supplementary Table 1). Next, 99 differentially expressed miRNAs were identified analyzing GC compared to AG (67 up-regulated and 32 down-regulated; Supplementary Table 2). The lowest number, 20 miRNAs, were found to be deregulated comparing AG and CON (6 up-regulated and 14 down-regulated; Supplementary Table 3). Differential expression results comparing GC *vs* GCaj, AG *vs* GCaj, and CON *vs* GCaj are presented in Supplementary Tables 4, 5 and 6 respectively.

Differential expression results and top five deregulated miRNAs in each case are represented in Figure 1A. Multidimensional scaling analysis of normalized expression values, assessing the similarity structure of miRNomes (Spearman's correlation distance), revealed 4 clusters, corresponding to the CON, AG, GC cancerous and adjacent tissues (Figure 1B). The AG cluster was intermediate between GC and CON, whereas GCaj was overlapping with AG and CON groups.

Hsa-miR-129-1-3p and hsa-miR-196a-5p may be employed for discrimination of healthy, premalignant, and malignant GC cases

To further study miRNome profiles, altered expression of miRNAs was analyzed in three main comparison groups: AG *vs* CON, GC *vs* CON and AG *vs* GC according to clinical significance. Analyzing uniquely deregulated miRNAs, 40 differentially expressed miRNAs were found when compared GC to CON (25.8% of all deregulated

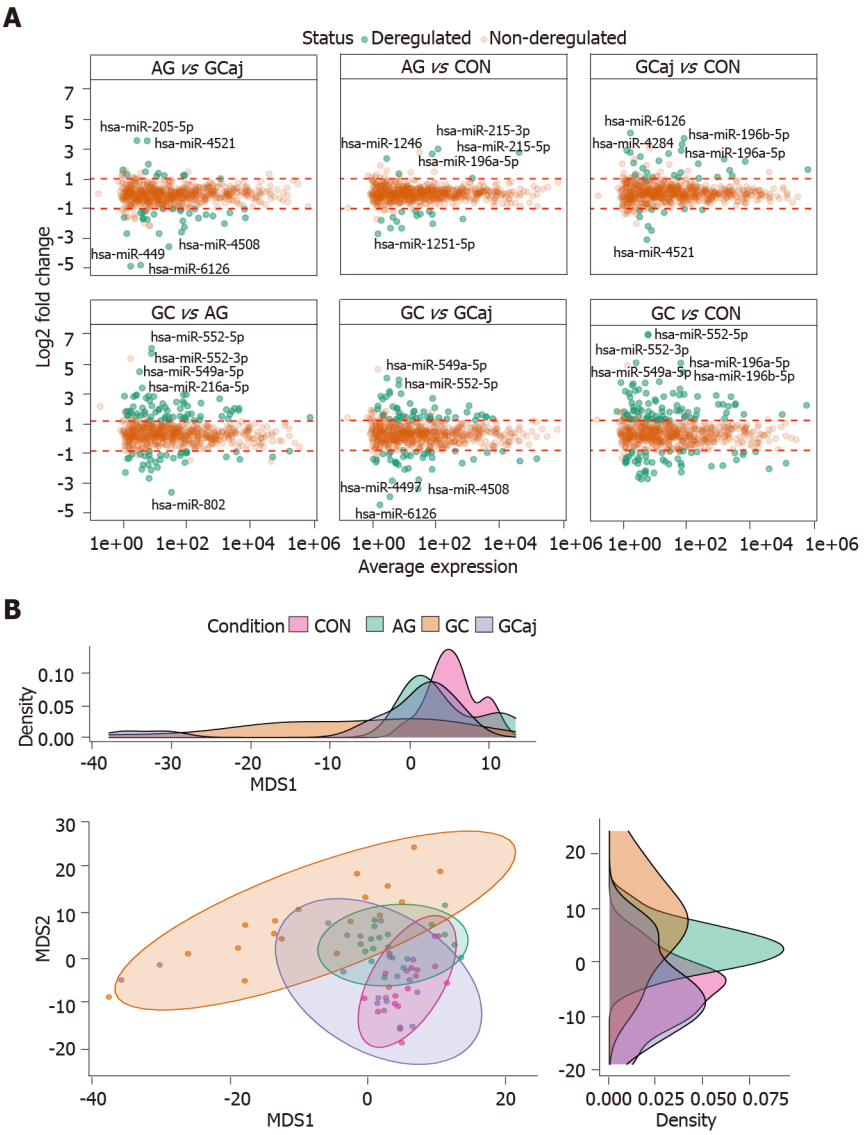


Figure 1 Results of microRNA differential expression analysis. A: Differentially expressed gastric tissue microRNAs among different conditions. *P*-adjusted < 0.05 and |log2 fold change| > 1; B: Multidimensional scaling plot based on normalized data showing a clustering corresponding to control, atrophic gastritis, gastric cancerous and adjacent tissues. The density plots show distributions of the first and second dimensions. CON: Control; AG: Atrophic gastritis; GC: Gastric cancerous; GCaj: Gastric adjacent tissue; MDS: Multidimensional scaling.

miRNAs), 18 (11.6%) - AG compared to GC, and 6 (3.9%) - AG compared to CON (Figure 2). Most of the deregulated miRNAs (n = 79, 68.7%) were similar between GC *vs* CON and GC *vs* AG comparison groups. 12 miRNAs (7.7%) were deregulated in both AG and GC groups when compared to CON. Four miRNAs (2.6%) were similarly deregulated between AG *vs* CON and AG *vs* GC groups. Finally, only 2 miRNAs (hsa-miR-129-1-3p and hsa-miR-196a-5p) (1.29%) were identified as deregulated between all comparison groups. AUC-ROC analysis revealed that expression level of hsa-miR-129-1-3p in tissues resulted in AUCs: 68.1%; 86.3%, and 78.1%, CON *vs* AG, CON *vs* GC, and AG *vs* GC, respectively (Figures 3A, 3B and 3C). In addition to this, expression level of hsa-miR-196a-5p could be significant for discrimination of CON *vs* AG, CON *vs* GC and AG *vs* GC and resulted in AUCs: 88.0%, 93.1% and 66.3% (Figures 3D, 3E and 3F).

Hsa-miR-129-1-3p and hsa-miR-196a-5p expression in the plasma follows the expression pattern of CON, AG, and GC tissues

Differential expression analysis of NGS data in tissue samples revealed that hsa-miR-129-1-3p was significantly down-regulated and hsa-miR-196a-5p was up-regulated in AG and GC tissues compared to CON (P = 0.002 and P = 0.00018; $P = 1.2 \times 10^{-5}$ and $P = 3.1 \times 10^{-5}$, respectively). Moreover, hsa-miR-129-1-3p was significantly down-regulated in the case of AG compared to GC (P = 0.0021) and reflected a stepwise process of a

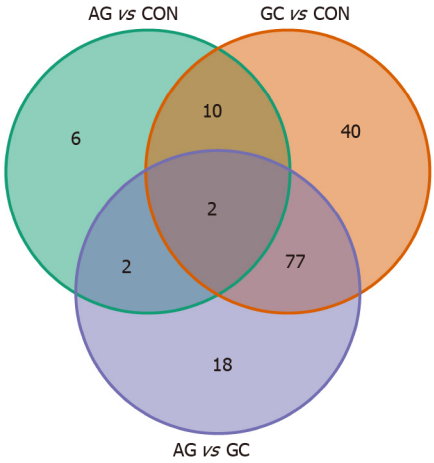


Figure 2 Venn diagram representing the number of commonly and uniquely differentially expressed microRNAs in three different comparison groups. *P*-adjusted < 0.05 and |log2 fold change| > 1. CON: Control; AG: Atrophic gastritis; GC: Gastric cancer.

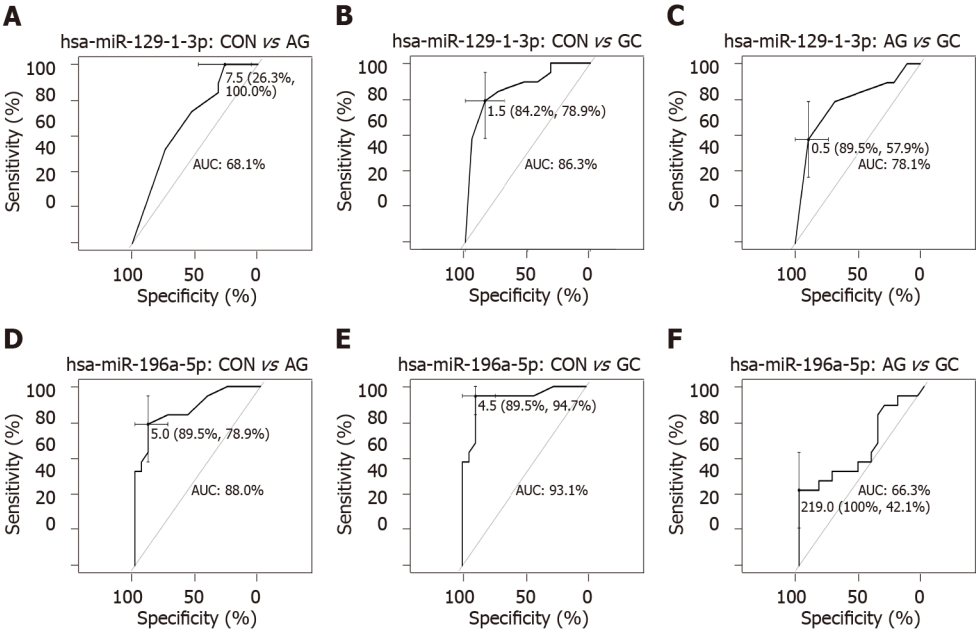


Figure 3 Receiver operating characteristic curves showing prediction performances of expression levels. A-C: Hsa-miR-129-1-3p; D-F: Hsa-miR-196a-5p in tissue samples between different comparison groups: Control *vs* atrophic gastritis; control *vs* gastric cancer; and atrophic gastritis *vs* gastric cancer. AUC: Area under the curve; CON: Control; AG: Atrophic gastritis; GC: Gastric cancer.

pathology (Figure 4A). Therefore, to identify whether the expression changes of these two miRNAs can be detected noninvasively in the body fluids of the patients, hsa-miR-129-1-3p and hsa-miR-196a-5p were selected for RT-qPCR analysis in plasma samples of independent cohort. The analysis showed similar expression patterns in the case of hsa-miR-129-1-3p, which was significantly down-regulated when comparing AG and GC groups (P = 0.024). There were no other significant findings between the groups (Figure 4B).

Hsa-miR-215-3p/5p and hsa-miR-934 may be associated with H. pylori-induced GC

To investigate role of miRNAs in AG atrophy progression (OLGA classification) and *H. pylori*-induced GC, differential miRNAs profile analysis in the subgroups of the study was performed. The analysis revealed a minor clustering in AG tissues corresponding to OLGA stages (Supplementary Figures 3A and 3H). *H. pylori* status in GC tissues (Supplementary Figure 3B). However, no significantly deregulated miRNAs were determined comparing I-II OLGA stages *vs* III-IV OLGA stages (AG tissue samples). On the other hand, analyzing GC group based on *H. pylori* infection status [*H. pylori* (neg.) *vs H. pylori* (pos.)], three miRNAs were shown to be significantly

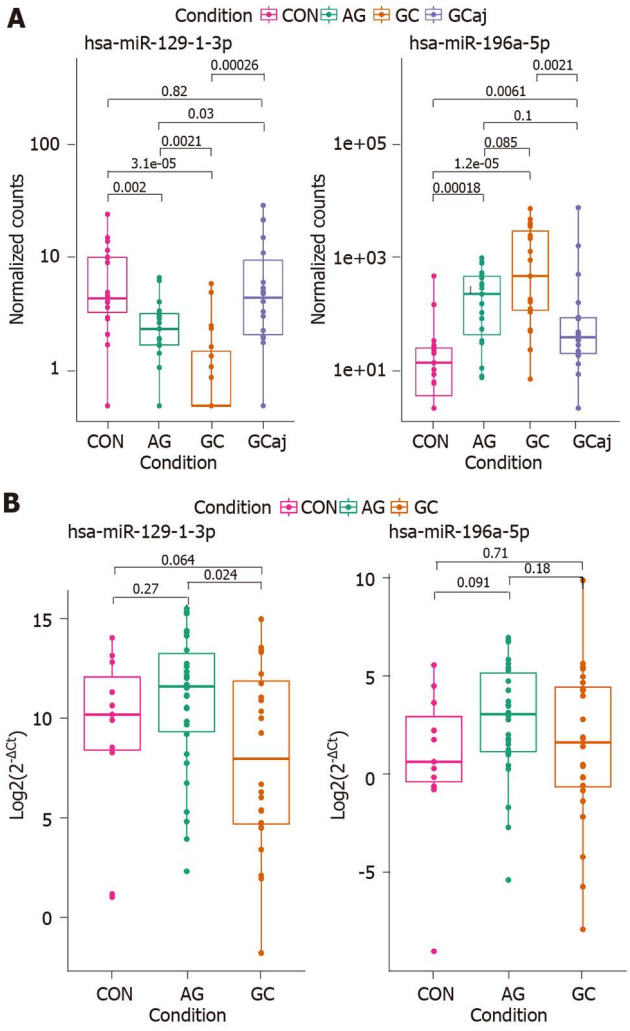


Figure 4 Hsa-miR-129-1-3p and hsa-miR-196a-5p expression levels in study comparison groups. A: Atrophic gastritis and gastric cancer tissue samples compared to controls; B: Atrophic gastritis and gastric cancer plasma samples compared to controls. Box plot graphs; boxes correspond to the median value and interquartile range. CON: Control; AG: Atrophic gastritis; GC: Gastric cancerous; GCaj: Gastric adjacent tissue.

deregulated: Hsa-miR-215-3p (log2FC = -4.52, *P*-adjusted = 0.02), hsa-miR-215-5p (log2FC = -4.00, *P*-adjusted = 0.02), and hsa-miR-934 (log2FC = 6.09, *P*-adjusted = 0.02).

DISCUSSION

This study represents comprehensive miRNome profiling of premalignant and malignant GC cases by implementing high throughput technologies such as NGS. Although there are several studies reporting profiles of GC tissue miRNAs[23,24], analysis of the association between miRNA expression and AG is very scarce reporting only individual miRNAs[25]. Moreover, based on small RNA-seq findings, two miRNAs were analyzed in subjects' plasma samples to investigate potential non-invasive markers. To our best knowledge this is the first study analyzing AG and GC tissue miRNomes in the subjects of European origin.

First, our study showed different profiles of deregulated miRNAs between tissue samples of studied groups. In total, 20 differentially expressed miRNAs were identified in AG and 129 - in GC comparing to CON; also 99 deregulated miRNAs - comparing GC and AG groups. MiRNAs such as hsa-miR-3131, hsa-miR-483, hsa-miR-150, hsa-miR-200a-3p, hsa-miR-873-5p were previously reported by the GC profiling studies of Pereira *et al*[23] and Assumpção *et al*[24]. Yet, we were able to identify number of novel miRNAs (of which hsa-miR-548ba, hsa-miR-4521, hsa-miR-549a were the most deregulated). There are no data showing the role of these novel miRNAs in inflammatory or tumorous processes of gastric tissue. However, recent studies have shown that hsa-miR-548ba was associated with bladder cancer, hsa-miR-549a with the

metastasis of renal cancer, and hsa-miR-4521 with *H. pylori* infection in esophageal epithelial cells[26-28]. Taking into consideration miRNome of AG, hsa-miR-3591-3p, hsa-miR-122-3p and hsa-miR-122-5p, hsa-miR-451a miRNAs were already reported by Liu *et al*[29], while the most deregulated miRNAs including hsa-miR-215, hsa-miR-4497, and hsa-miR-1251 were reported for the first time in our study. Previous research showed that hsa-miR-215-5p was deregulated in different lesions of the gastrointestinal tract (Barrett's esophagus, intraepithelial neoplastic lesions, ulcerative colitis)[30-32]. However, hsa-miR-4497 and hsa-miR-452 were not previously associated with AG but were reported to play an important role in GC development[33,34].

Next, we identified hsa-miR-215-3p and hsa-miR-215-5p to be down-regulated while hsa-miR-934 - up-regulated in GC group comparing negative and positive *H. pylori* infection status. Studies revealed the altered expression of various miRNAs in *H. pylori*-induced GC tissue samples, including miR-934, miR-146a, miR-375, miR-204[35-37]. Although, hsa-miR-215 deregulation was previously associated with GC[38-40], there is no data showing its link with *H. pylori* infection.

In addition to this, we showed that two miRNAs (hsa-miR-129-1-3p and hsa-miR-196a-5p) were gradually deregulated comparing all three study groups (CON, AG, and GC) which also corresponds to pathological cascade of GC. In concordance to our results, it has already been shown that hsa-miR-129-1-3p was down-regulated in GC tissues, function as a tumor suppressor in GC and even corresponds to the same expression pattern in gastric juice[41,42]. There is no data regarding the hsa-miR-196a expression in AG tissue, however, investigators have revealed that hsa-miR-196a is overexpressed in GC tissue, plasma, commercial cell lines and promotes cell proliferation[43,44]. ROC-AUC analysis suggests great potential of hsa-miR-196a-5p expression in tissue for discrimination of AG and GC in contrast to CON (AUC = 89.5% and AUC = 89.5%, respectively). Therefore, further studies are needed to confirm this finding.

Finally, selected miRNAs were analyzed in independent cohort of CON, AG, and GC plasma samples by using RT-qPCR. Results showed similar deregulation direction in plasma samples as in the tissue samples. However, significant differences were only determined comparing the expression of hsa-miR-129-1-3p between AG and GC suggesting its potential role in non-invasive diagnostics of malignant cases. No significant expression changes were observed between study groups and hsa-miR-196a-5p. Other studies have shown controversial results: Tsai *et al*[45] reported that miR-196a/b was up-regulated in both the plasma and tissue of metastatic GC patients, while miRNome profiling study revealed that miR-196a-5p was found to be down-regulated in plasma of patients with precursor lesions of GC compared to non-active gastritis[46].

In our study, using NGS and RT-qPCR techniques we have shown the distinct miRNome profiles of CON, AG, GC, GCaj tissues, and potential of specific miRNAs as non-invasive biomarkers. In addition to this, novel miRNAs not previously reported as AG or GC associated epigenetic markers were identified. We have shown that hsa-miR-196a-5p expression in tissue could be significant for discrimination between CON and AG or GC, confirmed hsa-miR-129-1-3p as non-invasive biomarker in disease progression monitoring, and showed that miRNAs could be a great candidate for future research of new diagnostic approaches.

CONCLUSION

In conclusion, we showed gradual deregulation of hsa-miR-196a-5p and hsa-miR-129-1-3p in the gastric carcinogenesis pathway and confirmed hsa-miR-129-1-3p as a possible non-invasive biomarker. We also found hsa-miR-215-3p/5p and hsa-miR-934 to be significantly deregulated in GC based on *H. pylori* infection status. These data provide novel insights into complex GC pathogenesis cascade which could be highly significant for future studies of new diagnostic GC targets.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a complex disease arising from the interaction of environmental (*e.g.*, diet, smoking, *etc.*) and host-associated factors [*e.g.*, *Helicobacter pylori* (*H. pylori*) infection, genetics, *etc.*]. Due to its silent course, it is also one of the most lethal cancers

worldwide as it is usually diagnosed at the advanced stages.

Research motivation

Novel biomarkers that would help to improve GC patients' diagnosis and prognosis are highly needed. Studies show that microRNAs (miRNAs) play an important role in many cancers and could be a promising biomarker or even therapeutic target.

Research objectives

The objectives of the study were to analyze whole miRNome profiles of control, premalignant and malignant gastric tissues, and select the potential miRNA markers that could have a potential for minimally invasive GC diagnostics.

Research methods

Total RNA from gastric tissue samples was subjected for small RNA sequencing (smRNA-seq). Plasma total circulating nucleic acids were used for the expression analysis of the most tissue deregulated miRNAs by real-time quantitative polymerase chain reaction. Statistical analysis involved the differential expression and discrimination analyses.

Research results

The abundance of altered expression miRNAs corresponded to a pathological cascade of GC development. Hsa-miR-129-1-3p and has-miR-196a-5p were shown to be deregulated in healthy-premalignant-malignant sequence. In addition to this, we showed that down-regulation of hsa-miR-129-1-3p could also be detected non-invasively in GC patients' plasma samples. Finally, results indicated that hsa-miR-215-3p/5p and hsa-miR-934 were significantly deregulated based on *H. pylori* infection status for GC patients.

Research conclusions

Gastric tissue miRNome study provides extensive profiling of control, premalignant and malignant cases. Based on smRNA-seq results several miRNAs were shown as potential gastric carcinogenesis (hsa-miR-196a-5p and hsa-miR-129-1-3p); and *H. Pylori* -related (hsa-miR-215-3p/5p and hsa-miR-934) biomarkers.

Research perspectives

This study provides novel insights into complex GC pathogenesis cascade and could serve as a reference for future research to support our findings.

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ORIGINAL ARTICLE

Retrospective Study

Validation of the PAGE-B score to predict hepatocellular carcinoma risk in caucasian chronic hepatitis B patients on treatment

Pinar Gokcen, Fatih Guzelbulut, Gupse Adali, Ayca Gokce Degirmenci Salturk, Oguzhan Ozturk, Ozgur Bahadir, Emine Kanatsiz, Mevlut Kiyak, Kamil Ozdil, Hamdi Levent Doganay

ORCID number: Pinar Gokcen 0000-0002-6742-6976; Fatih Guzelbulut 0000-0003-4889-208X; Gupse Adali 0000-0003-2157-0304; Ayca Gokce Degirmenci Salturk 0000-0002-1979-9145; Oguzhan Ozturk 0000-0002-7718-8684; Ozgur Bahadir 0000-0002-9472-5170; Emine Kanatsiz 0000-0003-2971-6443; Mevlut Kiyak 0000-0003-1672-5914; Kamil Ozdil 0000-0003-2556-3064; Hamdi Levent Doganay 0000-0002-2263-6689.

Author contributions: Doganay HL and Guzelbulut F equally contributed to this work. Doganay HL, Gokcen P, and Guzelbulut F contributed to the concept; Doganay HL, Gokcen P, Adali G, and Guzelbulut F designed this study; Doganay HL and Guzelbulut F supervised this study; Gokcen P, Kiyak M, Kanatsiz E, Bahadir O, Ozturk O and Ozdil K prepare the materials; Gokcen P, Guzelbulut F, Degirmenci Salturk AG, Kanatsiz E, Kiyak M, Ozturk O and Ozdil K collected and/or processed the data; Gokcen P, Doganay HL, Guzelbulut F and Adali G analyzed and/or interpreted the data; Gokcen P, Guzelbulut F., Kanatsiz E, Kiyak M, Degirmenci Salturk AG and Bahadir O searched the literature; Gokcen P, Doganay HL, Adali G and

Pinar Gokcen, Gupse Adali, Oguzhan Ozturk, Kamil Ozdil, Hamdi Levent Doganay, Department of Gastroenterology, Health Sciences University, Umraniye Teaching and Research Hospital, Istanbul 34764, Turkey

Fatih Guzelbulut, Ayca Gokce Degirmenci Salturk, Ozgur Bahadir, Emine Kanatsiz, Mevlut Kiyak, Department of Gastroenterology, Health Sciences University, Haydarpasa Numune Teaching and Research Hospital, Istanbul 34668, Turkey

Corresponding author: Hamdi Levent Doganay, MD, Professor, Department of Gastroenterology, Health Sciences University, Umraniye Teaching and Research Hospital, No. 1 Adem Yavuz Street, Elmalikent, Umraniye, Istanbul 34764, Turkey.
ldoganay@hotmail.com

Abstract

BACKGROUND

Several risk scores have been developed to predict hepatocellular carcinoma (HCC) risk in chronic hepatitis B (CHB) patients. The majority of risk scores are based on pretreatment variables that are no longer considered risk factors for HCC development due to the suppression of hepatitis B virus replication early in the course of potent antiviral treatment in most patients. The PAGE-B score, which is based on platelet levels, age and sex, has been shown to accurately predict HCC risk in CHB patients on antiviral treatment in various populations.

AIM

We aimed to evaluate the PAGE-B score in predicting HCC risk in Turkish CHB patients on antiviral treatment.

METHODS

In this study, we recruited 742 CHB patients who had been treated with tenofovir disoproxil fumarate or entecavir for ≥ 1 year. Risk groups were determined according to the PAGE-B scores as follows: ≤ 9, low; 10-17, moderate and ≥ 18, high. The cumulative HCC incidences in each risk group were computed using Kaplan-Meier analysis and were compared using the log-rank test. The accuracy of the PAGE-B score in predicting HCC risk was evaluated using a time-dependent area under the receiver operating characteristic (AUROC) curve at all study time points. Univariate and multivariate logistic regression analyses were

Guzelbulut F wrote the Manuscript; Doganay HL, Guzelbulut F and Adali G contributed to review critical.

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used to assess the risk factors for HCC development.

RESULTS

The mean follow-up time was 54.7 ± 1.2 mo. HCC was diagnosed in 26 patients (3.5%). The cumulative HCC incidences at 1, 3, 5 and 10 years were 0%, 0%, 0% and 0.4% in the PAGE-B low-risk group; 0%, 1.2%, 1.5% and 2.1% in the PAGE-B moderate-risk group; and 5%, 11.7%, 12.5%, and 15% in the PAGE-B high-risk group, respectively (log-rank $P < 0.001$). The AUROCs of the PAGE-B score in the prediction of HCC development at 1, 3, 5 and 10 years were 0.977, 0.903, 0.903 and 0.865, respectively. In the multivariable analysis, older age, male sex, lower platelet levels, presence of cirrhosis, and absence of alanine aminotransferase normalization at month 6 were associated with HCC development (all $P < 0.05$).

CONCLUSION

The PAGE-B score is a practical tool to predict HCC risk in Turkish patients with CHB and may be helpful to improve surveillance strategies.

Key Words: Chronic hepatitis B; Hepatocellular carcinoma; PAGE-B score; Surveillance

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Core Tip: We evaluated the accuracy of the PAGE-B score in predicting hepatocellular carcinoma (HCC) risk in Turkish patients with chronic hepatitis B on antiviral treatment. The cumulative HCC incidences at 5 and 10 years were 0% and 0.4%, 1.5% and 2.1%, and 12.5% and 15.0% in the low-, moderate- and high-risk groups based on the PAGE-B score, respectively. The area under the receiver operating characteristics of the PAGE-B score in the prediction of HCC risk at 5 and 10 years were 0.903 and 0.865, respectively. The PAGE-B score was found to be highly negative predictive and reliable for a cutoff value of ≤ 9 in predicting HCC development.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 7th most prevalent among all cancers and ranks 4th in cancer-related mortality[1]. Chronic hepatitis B (CHB) virus infection affects 257 million people worldwide and is one of the most common etiologies of HCC, accounting for 33% of HCC-related mortality[2]. Nucleos(t)ide analogs suppress hepatitis B virus (HBV) replication in most patients; however, the risk of HCC persists even in patients with suppressed viral replication. Treatment options for advanced-stage HCC are quite limited, and the 5-year survival rate is 18.1%[3]. Therefore, identifying patients who are at high risk for HCC development and detecting tumors at early stages are crucial. Recent guidelines recommend HCC surveillance with ultrasound (USG) twice a year in patients who are at high risk for HCC development [4]. However, not all patients with CHB have the same risk for HCC. There is an ongoing need for a scoring system to predict HCC risk that offers an easy application in clinical practice and a high predictive value to perform effective surveillance in high-risk patients and eliminate unnecessary surveillance in low-risk patients.

Various risk scores have been developed to identify CHB patients at high risk for HCC development. However, many of the risk scores for HCC have focused on untreated patients, and they are mostly based on pretreatment risk factors for HCC, such as hepatitis B e antigen (HBeAg) status, serum HBV DNA, alanine aminotransferase (ALT), albumin and bilirubin levels[5,6]. Most of the baseline virological factors are no longer considered risk factors for HCC, as HBV replication is suppressed early

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in the majority of patients receiving potent antiviral treatment. Therefore, risk scores that include parameters not easily modified by treatment are needed. Among these, the PAGE-B is a practical risk score that includes platelet count, age and sex and has been validated in various patient populations[7-9]. In this study, we aimed to evaluate the accuracy of the PAGE-B score in predicting HCC risk in Turkish CHB patients on tenofovir disoproxil fumarate (TDF) or entecavir (ETV) therapy.

MATERIALS AND METHODS

Patient population and Follow-up Definitions

Medical records of CHB patients who were on follow-up in hepatology outpatient clinics at Umraniye Training and Research Hospital (Istanbul, Turkey) and Haydarpaşa Numune Training and Research Hospital (Istanbul, Turkey) between January 2007 to December 2018 were retrospectively evaluated. The inclusion criteria were as follows: Age ≥ 16 years, HBsAg positivity for ≥ 6 mo, and treatment with TDF or ETV for at least 12 mo. The exclusion criteria were as follows: Age < 16 years, decompensated cirrhosis, having HCC diagnosis before or during the first 6 mo of therapy, history of liver transplantation, and coinfection with hepatitis C virus, hepatitis D virus or human immunodeficiency virus.

Laboratory tests, including HBeAg, anti-HBe, HBV DNA, aspartate aminotransferase, ALT, albumin, bilirubin and alpha-fetoprotein (AFP) levels, international normalized ratio, and complete blood count at the start of therapy and during follow-up at 3-6 mo intervals, were recorded. The results of imaging studies, *e.g.*, USG, triphasic computed tomography (CT) and dynamic contrast-enhanced magnetic resonance imaging (MRI), at the start of therapy and during follow-up were recorded. The presence of comorbidities and liver biopsy results, if available, were also recorded. Virological response was defined as a serum HBV DNA level < 80 IU/mL. Maintained virological response was defined as serum HBV DNA negativity without subsequent positivity. A biochemical response was achieved when the serum ALT level dropped below 42 U/L. Hepatic flare was defined as an elevation of ALT ≥ 2 × upper limit of normal with subsequent HBV DNA positivity in patients with virological response. Liver biopsies were evaluated according to the ISHAK staging system[10]. Patients with pretreatment fibrosis scores between 0 and 4 (F0-4) were considered noncirrhotic. Patients with fibrosis scores 5 and 6 (F5-6) or those with radiological (nodular appearance of liver surface, parenchymal thickening, caudate lobe enlargement, portal vein diameter > 13 mm) or endoscopic (varices, portal gastropathy) findings of cirrhosis were considered compensated cirrhotic. Decompensated cirrhosis was defined as the presence of ascites, variceal bleeding or hepatic encephalopathy. Patients underwent HCC surveillance with abdominal USG at 6-12 mo intervals. In the presence of suspicious lesions on USG, cross-sectional imaging with triphasic CT and/or dynamic contrast-enhanced MRI were performed. A diagnosis of HCC was made following the current guidelines[5]. The PAGE-B score included the parameters of platelet count, age, and sex. Scoring was performed as follows: (1) For age 16-29 years, 0 points; 30-39 years, 2 points; 40-49 years, 4 points; 50-59 years, 6 points; 60-69 years, 8 points; ≥ 70 years, 10 points; (2) For female gender, 0 points and for male gender, 6 points; and (3) For platelet count (/mm^3) ≥ 200000, 0 points; 100000-199999, 6 points; < 100000, 9 points. The score ranged from 0-25 points. Based on their PAGE-B scores, patients were classified as ≤ 9, low risk; 10-17, moderate risk; and ≥ 18, high risk [7].

Statistical analysis

Statistical data were analyzed using SPSS v.23.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics are presented as the mean ± standard error of the mean for continuous variables. Variables were tested for normality using the Kolmogorov-Smirnov test. Independent *t*-tests were used to compare parametric variables, and chi-squared tests, continuity correction, or Fisher's exact tests were used to compare categorical variables. The cumulative effect of PAGE-B risk groups on survival was assessed using the log-rank test. Survival rates were computed by Kaplan-Meier survival analysis. Accuracy in predicting HCC occurrence was evaluated using a time-dependent area under the receiver operating characteristic (AUROC) curve at all study time points. Univariate and multivariate logistic regression analysis models were used to determine the effects of the variables on the risk of developing HCC. Cirrhosis and platelet count were analyzed separately in logistic regression model as they showed collinearity. Tests were interpreted at a 95% confidence interval. A *P* value ≤ 0.05 was

Table 1 Demographic and follow-up characteristics of study population

	***n* = 742**
Age, yr ± SE	45.0 ± 0.5
Gender, male *n* (%)	472 (63.6)
Follow-up, mo ± SE	54.7 ± 1.2
Diabetes mellitus, *n* (%)	116 (15.7)
HBeAg positivity, *n* (%)	171 (23.0)
Cirrhosis, *n* (%)	161 (21.7)
NA(s) before ETV/TDF, *n* (%)	162 (21.8)
Antiviral treatment (ETV/TDF), *n* (%)	240 (32.3)/502(67.7)
MVR, *n* (%)	633 (85.3)
Hepatic flare, *n* (%)	25 (3.4)
ALT normalization at 6 mo, *n* (%)	620 (85.9)
Virological response at 6 mo, *n* (%)	597 (85.4)
PAGE-B score ± SE	11.1 ± 0.2
PAGE-B score-risk groups, *n* (%)	
Low	281 (37.9)
Moderate	341 (46)
High	120 (16.2)
HCC cases during follow up, *n* (%)	26 (3.5)

HBeAg: Hepatitis B e antigen; ALT: Alanine transaminase; ETV: Entecavir; HCC: Hepatocellular carcinoma; MVR: Maintained virological response; NA(s): Nucleos(t)ide analogue(s); TDF: Tenofovir disoproxil fumarate; SE: Standard error of mean.

considered statistically significant.

The study was approved by the local ethics committees of Umraniye Training and Research Hospital and Haydarpasa Numune Training and Research Hospital.

RESULTS

A total of 742 patients were enrolled in the study. The mean age was 45.0 ± 0.5 (17-93) years, and 472 (63.6%) patients were male. One hundred and sixty-one patients (21.7%) had cirrhosis. Of the total patients, 502 (67.7%) received TDF, and 240 (32.3%) received ETV. One hundred and sixty-two (21.8%) of patients were lamivudine-experienced. At month 12, 597 patients (85.4%) achieved virological response, and 620 patients (85.9%) achieved ALT normalization. Twenty-five (3.4%) patients had hepatic flare. The mean follow-up time was 54.7 ± 1.2 (5-145) mo. The demographic and clinical characteristics and follow-up data of the patients are presented in Table 1.

During the follow-up period, 26 patients (3.5%) developed HCC. Patients who developed HCC were older, male predominant, had lower albumin and platelet levels, and had higher AFP levels than those who did not develop HCC (all $P < 0.05$). Cirrhosis and diabetes mellitus were more common in patients who developed HCC than in those who did not develop HCC (both $P < 0.05$) (Table 2).

In the univariable analysis, older age, male sex, lower platelet levels, presence of diabetes mellitus, presence of cirrhosis, absence of ALT normalization at month 6, and pretreatment AFP levels were associated with HCC development (all $P < 0.05$). HCC was not detected in any patients with hepatic flare. In the multivariable analysis, older age [odd ratio (OR) = 1.1; 95% confidence interval (CI): 1.0-1.1], male sex (OR = 8.9; 95%CI: 1.1-70.7), lower platelet levels (OR = 1.0; 95%CI: 1.0-1.0), presence of cirrhosis (OR = 3.1; 95%CI: 1.1-8.2), and absence of ALT normalization at month 6 (OR = 0.2; 95%CI: 0.1-0.7) were associated with HCC occurrence (all $P < 0.05$) (Table 3).

Table 2 Comparison of baseline characteristics of patients with and without hepatocellular carcinoma

	Patients with HCC	Patients without HCC	*P* value
Age (yr) mean ± SE	57.8 ± 2.3	44.5 ± 0.5	< 0.001
Male gender, *n* (%)	24 (92.3)	448 (62.6)	0.004
Cirrhosis, *n* (%)	16 (61.5)	145 (20.3)	< 0.001
Diabetes mellitus, *n* (%)	8 (32)	108(15.1)	0.043
Antiviral treatment (ETV/TDF), *n* (%)	10 (38.5)/16 (61.5)	230 (32.1)/486 (67.9)	0.642
Laboratory (mean ± SE)			
HBeAg positivity, *n* (%)	6 (23.1)	165 (23.0)	1.000
ALT (IU/L)	92.9 ± 25.7	98.3 ± 5.7	0.856
Albumin (g/dL)	3.9 ± 0.1	4.1 ± 0.0	0.014
Total bilirubin (mg/dL)	0.9 ± 0.1	1.0 ± 0.1	0.709
AFP (ng/mL)	23.3 ± 9.6	5.2 ± 0.4	< 0.001
INR	1.1 ± 0.0	1.1 ± 0.0	0.143
Platelet (10^3/mL)	128.8 ± 8.6	203.5 ± 2.5	< 0.001
HBV-DNA (log IU/mL)	5.4 ± 0.3	5.5 ± 0.1	0.859

ALT: Alanine transaminase; AFP: Alpha-fetoprotein; ETV: Entecavir; HCC: Hepatocellular carcinoma; SE: Standard error of mean; TDF: Tenofovir disoproxil fumarate; HBeAg: Hepatitis B e antigen; INR: International normalized ratio; HBV: Hepatitis B virus.

Validation of PAGE-B risk score

The mean PAGE-B score was 11.1 ± 0.2. According to the PAGE-B score, 281 (37.9%), 341 (46%) and 120 (16.2%) patients had low-risk, moderate-risk and high-risk of HCC development, respectively. Nineteen (6.8%), 78 (22.9%) and 64 (53.3%) patients had cirrhosis in the low-, moderate- and high-risk groups, respectively ($P < 0.001$). One (0.4%), 7 (2.1%) and 18 (15%) patients developed HCC in the low-, moderate- and high-risk groups, respectively ($P < 0.001$). For a PAGE-B score cutoff value ≤ 9, the sensitivity, specificity, positive and negative predictive values for the prediction of HCC were 96.2%, 39.1%, 5.4% and 99.6%, respectively. The AUROCs of the PAGE-B score in the prediction of HCC risk at 1, 3, 5 and 10 years were 0.977, 0.903, 0.903 and 0.865, respectively (Figure 1). The cumulative HCC incidences at 1, 3, 5 and 10 years were 0%, 0%, 0% and 0.4%, respectively, in the PAGE-B low-risk group; 0%, 1.2%, 1.5% and 2.1%, respectively, in the PAGE-B moderate-risk group; and 5.0%, 11.7%, 12.5%, and 15.0%, respectively, in the PAGE-B high-risk group (log-rank $P < 0.001$) (Figure 2).

DISCUSSION

The ultimate goal of CHB therapy is to extend the survival of patients by preventing progression to cirrhosis, HCC development and the need for transplantation. This objective has been achieved substantially with the widespread use of TDF and ETV, which have a high genetic barrier to resistance. However, the risk of HCC is not eliminated despite effective antiviral drugs. Various studies have aimed to evaluate the risk of HCC development in various populations using risk scores that include clinical or laboratory parameters. The REACH-B (age, sex, HBsAg status, and HBV DNA concentration) score was the first scoring system that did not include cirrhosis as a parameter, and it was associated with a 5-year HCC incidence of 2.6% in the low-risk group[11]. The GAG-HCC (age, sex, HBV DNA, core promoter mutations and cirrhosis) and CU-HCC (age, viral load, bilirubin, albumin, cirrhosis) scores have a negative predictive value of 98.3% for 5-year HCC incidence in treatment-naive Asian patients[12]. These scoring systems, which were validated in untreated patients, were less predictive when applied to patients on antiviral treatment[13]. For example, a high serum HBV DNA level, which is included in REACH-B, is no longer regarded as a risk factor for HCC with the use of potent antivirals[8]. Moreover, detection of core promoter mutations that are included in the GAG-HCC scoring system is not always

Table 3 Risk factors associated with the risk of hepatocellular carcinoma development

	Univariate analysis, OR (95% CI)	*P* value	Multivariate analysis, OR (95% CI)	*P* value
Age (per yr increase)	1.1 (1.0-1.1)	< 0.001	1.1 (1.0-1.1)	< 0.001
Gender (male *vs* female)	7.2 (1.7-30.6)	0.004	8.9 (1.1-70.7)	0.038
Platelet[1] (10^3/mL)	1.0 (1.0-1.0)	< 0.001	1.0 (1.0-1.0)	< 0.001
AFP (ng/mL)	1.0 (1.0-1.0)	< 0.001	1.0 (1.0-1.0)	0.141
HBeAg status (positive *vs* negative)	1.0 (0.4-2.5)	1.000		
Diabetes mellitus (yes *vs* no)	2.7 (1.1-6.3)	0.043	0.6 (0.2-1.7)	0.308
NA(s) before ETV/TDF (yes *vs* no)	2.0 (0.9-4.5)	0.143		
Cirrhosis[1] (yes *vs* no)	6.3 (2.8-14.2)	< 0.001	3.1 (1.1-8.2)	0.026
Antiviral treatment (ETV *vs* TDF)	0.8 (0.3-1.7)	0.642		
MVR (no *vs* yes)	0.6 (0.2-1.4)	0.253		
ALT normalization at month 6 (no *vs* yes)	0.4 (0.2-0.9)	0.043	0.2 (0.1-0.7)	0.009
ALT normalization at month 12 (no *vs* yes)	0.4 (0.2-1.0)	0.101		
Virological response at month 6 (no *vs* yes)	0.8 (0.3-1.9)	0.622		
Virological response at month 6 (no *vs* yes)	0.7 (0.2-2.2)	0.530		

[1]Cirrhosis and platelet count were analyzed separately among with other independent variables in logistic regression model as they showed collinearity. AFP: Alpha-fetoprotein; HBeAg: Hepatitis B e antigen; CI: Confidence interval; ETV: Entecavir; MVR: Maintained virological response; NA(s): Nucleos(t)ide analogue(s); TDF: Tenofovir disoproxil fumarate.

possible. To solve these problems, novel scoring systems were developed in patients on treatment. The PAGE-B (age, sex and platelet count), CAGE-B (age, presence of baseline cirrhosis), SAGE-B (age, liver stiffness measurements), CAMD (cirrhosis, age, male sex, diabetes mellitus) and HCC-RESCUE (age, sex, cirrhosis) scoring systems all have high negative predictive values for HCC development in their low-risk groups[7,14-16]. Among current scoring systems, the PAGE-B is the only one that does not include cirrhosis as a parameter. The presence of cirrhosis is the most important risk factor for HCC development, and the annual risk of HCC in patients with cirrhosis is 2.5%-4%[17]. Liver biopsy is the gold standard method for the diagnosis of cirrhosis. However, biopsy is associated with certain disadvantages, such as being an invasive method that can lead to potential complications, requiring tissue samples of an appropriate amount and from an appropriate localization, and producing false-negative results in the early period. Meanwhile, noninvasive methods that assess fibrosis, such as transient elastography, can produce operator-dependent false-positive results. It should also be emphasized that liver biopsy is not performed in patients with lamivudine, adefovir or telbivudine resistance prior to the start of new antiviral agents with a high genetic barrier to resistance. Therefore, these nucleos(t)ide analog-experienced patients may not have cirrhosis at the start of rescue therapy due to the resolution of cirrhosis after years of therapy. Our cohort also included 162 patients (21.8%) who had received lamivudine and developed resistance prior to the start of TDF/ETV treatment. Therefore, definitive confirmation of cirrhosis for all patients is impractical. To that point, the PAGE-B score has an advantage compared to the other scoring systems that include cirrhosis as a parameter. Supporting this, implementing ISHAK stage in the PAGE-B score did not improve the prediction of HCC risk[18].

In the present study, the incidence of HCC was determined to be 3.5%, and the cumulative HCC incidences at 5 years in the low-, moderate-, and high-risk groups were 0%, 1.5% and 12.5%, respectively. These rates were lower than those in the PAGE-B database (0%, 3%, 17%) but higher than those in Spain's CIBERHEP database (0%, 2.8%, 5%), which was used for the validation of the PAGE-B score. All three databases (ours, PAGE-B, CIBERHEP) had similar reliability of the PAGE-B score in the prediction of overall HCC development for a cutoff value ≥ 10[7,9]. It was thought

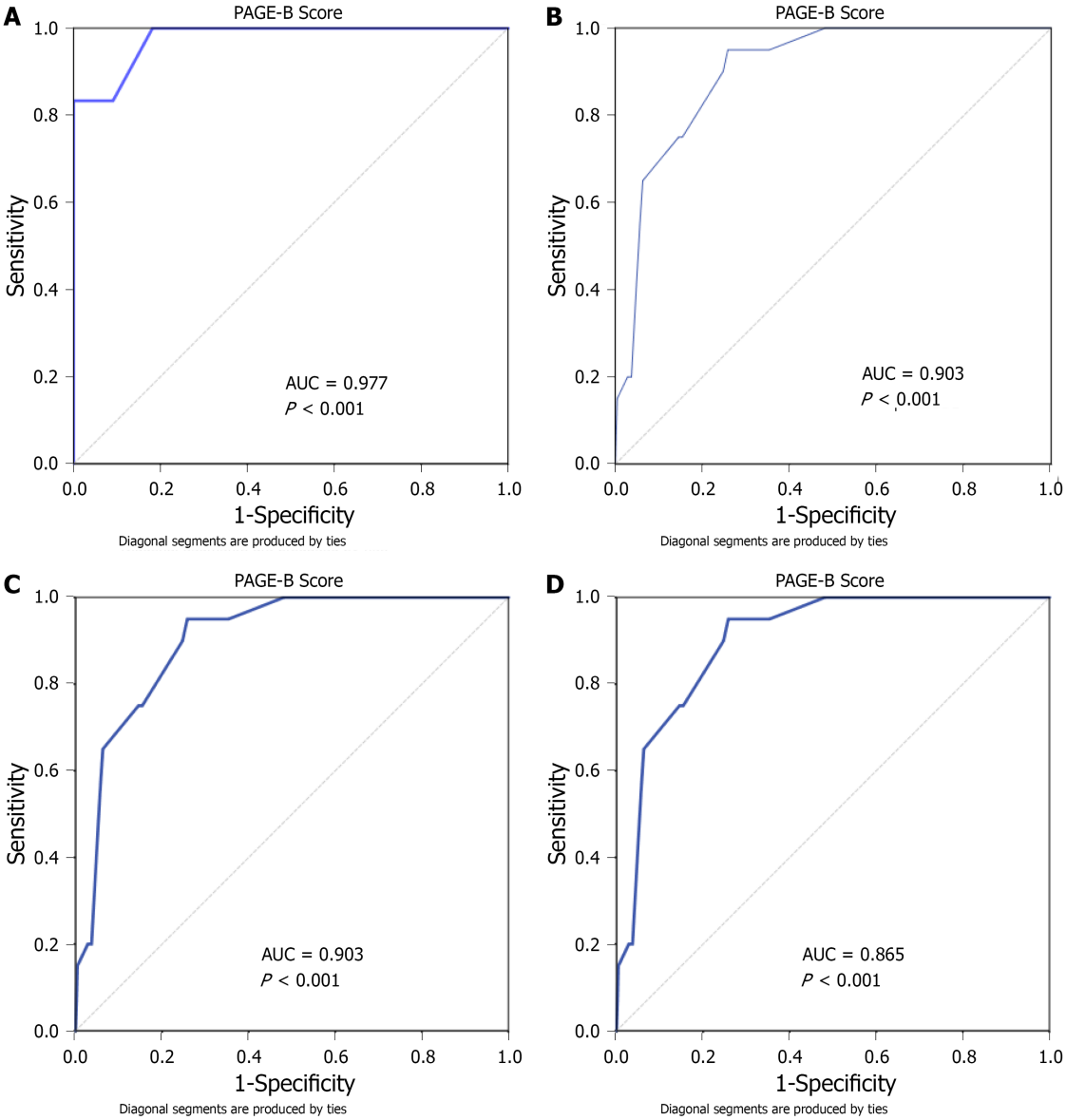


Figure 1 Receiver operating characteristic curves of the PAGE-B score for hepatocellular carcinoma development according to years. A: 1st year; B: 3rd year; C: 5th year; D: 10th year.

that the lower HCC rates in CIBERHEP may be related to the relatively low total number of patients and the number of patients who completed the 5-year follow-up [9]. The present study included 267 (36%) patients who had completed the 5-year follow-up, which was similar to the PAGE-B database; however, patients were younger on average than the PAGE-B database (45 *vs* 52). This might be a reason for the lower HCC incidence seen in this study. Additionally, the present study included mainly genotype D patients who are known to have less risk for HCC development, while genotypes A, B, D predominated in the PAGE-B database and genotype B and D in the CIBERHEP database.

In this study, we also evaluated 10-year HCC incidence. Although we did not find any cases of HCC in the low-risk group during the first 5 years of follow-up, one patient developed HCC at month 80. This 37-year-old noncirrhotic male patient was treated with ETV, did not have comorbidities, and had a PAGE-B score of 8. The study by Brouwer *et al*[18] showed that the estimated HCC incidences at 10 years in PAGE-B low-, moderate-, and high-risk groups were < 1.5%, 1.5%-17.5% and ≥ 17.5%, respectively, supporting our results (0.4%, 2.1%, 15%).

Current international guidelines recommend that patients with cirrhosis should undergo HCC surveillance systematically. However, there is no consensus about noncirrhotic CHB patients[19-22]. Diverging from other guidelines, the European Association for the Study of the Liver suggests that only patients with a PAGE-B score ≥ 10 in the noncirrhotic group should be included in screening[19]. In the present

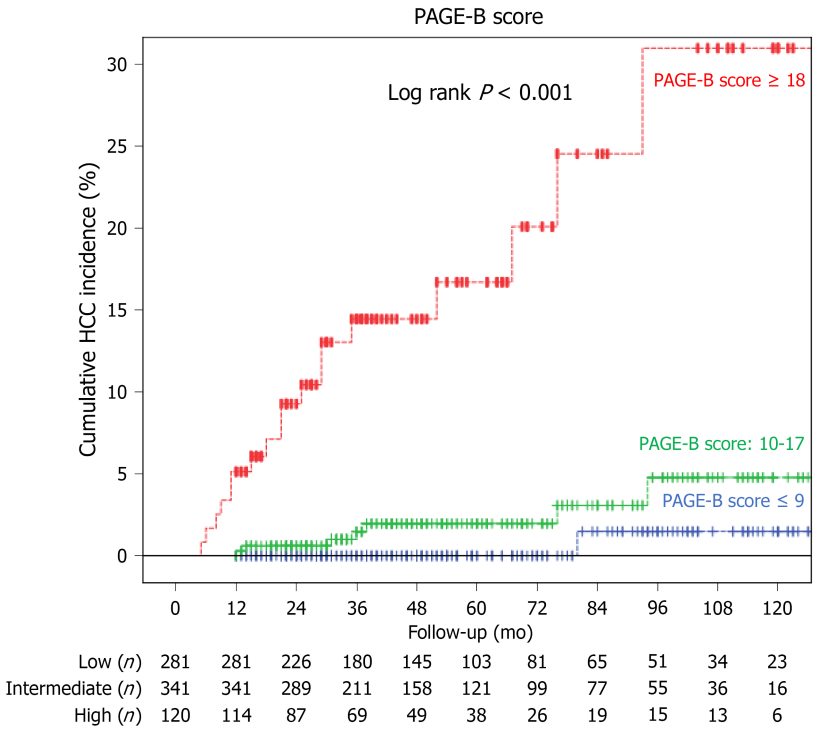


Figure 2 Cumulative hepatocellular carcinoma incidences according to PAGE-B risk scores. Low risk *vs* intermediate risk, log rank $P = 0.06$; low risk *vs* high risk, log rank $P < 0.001$; intermediate risk *vs* high risk, log rank $P < 0.001$.

study, unnecessary tests would be prevented in 281 (37.9%) patients with low-risk HCC by following this cost-effective approach.

The present study has some limitations. First, it is a retrospective study, so the effect of treatment non-compliance could not be determined precisely. Second, the patient number was relatively low. Third, only one-third of the patients completed the 5-year follow-up. Fourth, in Turkey, almost all patients are infected with genotype D virus (32), so no interpretation could be made for other genotypes.

CONCLUSION

PAGE–B successfully predicted patients who had a low risk for HCC during treatment with genetically high barrier antivirals. Ease of use without the need for biopsy or an impractical molecular test justifies implementing this score in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B (CHB) infection is an important health issue worldwide. Novel antiviral treatments lead to complete suppression of the virus and maintained suppression of viral replication prevents cirrhosis, decompensation in already cirrhotic patients and hepatocellular carcinoma (HCC). However, HCC risk is not totally eliminated and in pursuance of detecting cancer in early stages comprehensive follow up is needed. It is critical to stratify patients for risk predictions, especially to prevent unnecessary tests in low-risk patients.

Research motivation

Various risk scores have been developed to predict the development of HCC in CHB patients. The majority of studies on the risk scores had focused on untreated patients. Currently, almost all patients with CHB are treated with antiviral agents and better risk scores for patients under treatment is needed. The PAGE-B is a risk scoring system that includes platelet count, age and sex and has been validated in patients treated with antivirals.

Research objectives

We aimed to evaluate the accuracy of the PAGE-B scoring system in the prediction of HCC risk in CHB patients receiving entecavir (ETV) or tenofovir disoproxil fumarat therapy.

Research methods

We recruited 742 CHB patients who had been treated with tenofovir disoproxil fumarate or ETV for more than 1 year. Risk groups were determined according to the PAGE-B scores. We evaluated the accuracy of the PAGE-B score in predicting HCC.

Research results

HCC was diagnosed in 26 patients (3.5%) during 54.7 ± 1.2 mo mean follow up. The cumulative HCC incidences at 5 years were 0% in the PAGE-B low-risk group; 1.5% moderate-risk group; and 12.5%, in the high-risk group (log-rank $p < 0.001$). The AUROCs of the PAGE-B score in the prediction of HCC development at 5 years follow up was 0.903.

Research conclusions

PAGE–B had successfully predicted the patients who had a low risk of HCC during treatment with genetically high barrier antivirals.

Research perspectives

PAGE-B is a simple score that does not require biopsy or any impractical molecular test. The efficiency of PAGE-B justifies implementing this score in daily clinical practice.

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CASE REPORT

Gallbladder Burkitt's lymphoma mimicking gallbladder cancer: A case report

Kiyotaka Hosoda, Akira Shimizu, Koji Kubota, Tsuyoshi Notake, Hikaru Hayashi, Koya Yasukawa, Kentaro Umemura, Atsushi Kamachi, Takamune Goto, Hidenori Tomida, Shiori Yamazaki, Yuri Narusawa, Naoko Asano, Takeshi Uehara, Yuji Soejima

ORCID number: Kiyotaka Hosoda 0000-0001-6928-3676; Akira Shimizu 0000-0002-5015-1697; Koji Kubota 0000-0003-0164-7685; Tsuyoshi Notake 0000-0002-2277-5347; Hikaru Hayashi 0000-0002-3724-1751; Koya Yasukawa 0000-0002-3643-0921; Kentaro Umemura 0000-0003-1259-5438; Atsushi Kamachi 0000-0002-4012-0459; Takamune Goto 0000-0003-2410-0770; Hidenori Tomida 0000-0001-9278-7418; Shiori Yamazaki 0000-0001-8162-0620; Yuri Narusawa 0000-0003-0346-6220; Naoko Asano 0000-0001-7536-6179; Takeshi Uehara 0000-0002-7694-9015; Yuji Soejima 0000-0001-5539-9280.

Author contributions: Hosoda K, Kubota K, Notake T, Hayashi H, Umemura K, Kamachi A, Goto T, Tomida H, and Yamazaki S were the patient's hepato-biliary-pancreatic surgeons, they reviewed the literature and contributed to manuscript drafting; Narusawa Y, Asano N, and Uehara T were involved in pathology evaluation; Shimizu A and Soejima Y were responsible for revising the manuscript for important intellectual content; all the authors provided final approval for the version of the manuscript to be submitted.

Informed consent statement:

Kiyotaka Hosoda, Akira Shimizu, Koji Kubota, Tsuyoshi Notake, Hikaru Hayashi, Koya Yasukawa, Kentaro Umemura, Atsushi Kamachi, Takamune Goto, Hidenori Tomida, Shiori Yamazaki, Yuji Soejima, Division of Gastroenterological, Hepato-Biliary-Pancreatic, Transplantation and Pediatric Surgery, Department of Surgery, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Yuri Narusawa, Naoko Asano, Takeshi Uehara, Department of Laboratory Medicine, 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

Malignant lymphoma is a rare form of gallbladder malignancy. Most of these malignancies are diffuse large B-cell lymphomas or mucosa-associated lymphoid tissue-type lymphomas; however, Burkitt's lymphoma of the gallbladder is extremely rare, and only two previous reports are available in the literature. Herein, we report a rare case of Burkitt's lymphoma of the gallbladder mimicking gallbladder adenocarcinoma.

CASE SUMMARY

An 83-year-old man with no abdominal complaints was found to have a gallbladder tumor and periportal lymph node enlargement on computed tomography (CT) performed for hypertension screening. His laboratory data revealed slightly elevated serum levels of carcinoembryonic antigen and soluble interleukin 2 receptor. Imaging examinations revealed two irregular and contrast-enhanced masses extending into the gallbladder lumen, but these did not infiltrate the serosa. Moreover, a periportal lymph node had enlarged to 30 mm. Based on these findings, we diagnosed the patient as having gallbladder adenocarcinoma with lymph node metastasis, which was treated using bile duct resection with gallbladder bed resection and periportal lymph node dissection. However, the patient was finally diagnosed as having Burkitt's lymphoma. Although the surgical margin was pathologically negative, recurrence was noted at the hepatic

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radical margin and superior pancreaticoduodenal lymph nodes on positron emission tomography/CT soon after discharge. Thus, he was referred to a hematologist and started receiving treatment with reduced-dose cyclophosphamide, doxorubicin, vincristine, and prednisone.

CONCLUSION
Burkitt's lymphoma can occur in the gallbladder. Biopsy can be useful in cases with findings suggestive of gallbladder malignant lymphoma.

Key Words: Gallbladder; Malignant lymphoma; Burkitt's lymphoma; Gallbladder cancer; Lymphadenopathy; Case report

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Core Tip: Malignant lymphoma is a rare form of gallbladder malignancy, and Burkitt's lymphoma of the gallbladder is especially rare, with only two previous reports available in the literature. We report a case of gallbladder Burkitt's lymphoma that was preoperatively indistinguishable from gallbladder carcinoma. Unfortunately, the patient had lymphoma recurrence immediately after the surgery because of delayed chemotherapy initiation owing to postoperative complications due to an extended surgery. Although accurate preoperative diagnosis of gallbladder malignant lymphoma is quite difficult, some findings are suggestive of gallbladder malignant lymphoma, and hence, biopsy is recommended in these cases.

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INTRODUCTION

Malignant lymphoma of the gallbladder is extremely rare[1-4]. In almost all cases of this malignancy, patients are diagnosed as having gallbladder adenocarcinoma or cholecystitis at the time of surgery, and preoperative diagnosis is extremely difficult. Although several reports have documented malignant lymphomas of the gallbladder, most of these malignancies are diffuse large B-cell lymphomas and mucosa-associated lymphoid tissue-type lymphomas (MALTomas)[2,4,5], and only two reports have previously documented Burkitt's lymphoma of the gallbladder[6,7]. Herein, we report the case of a patient with Burkitt's lymphoma of the gallbladder mimicking gallbladder adenocarcinoma.

CASE PRESENTATION

Chief complaints

An 83-year-old man with no abdominal complaints was found to have a gallbladder tumor along with periportal lymph node enlargement and was admitted to our institution for further investigation.

History of present illness

The tumor was detected during contrast-enhanced computed tomography (CT) performed for a detailed examination of hypertension.

History of past illness

The patient had a history of cerebral artery stenosis and paroxysmal atrial fibrillation.

S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

Personal and family history

No personal and family history.

Physical examination

Abdominal examination revealed no palpable mass or tenderness.

Laboratory examinations

Laboratory examinations performed upon admission revealed mild to moderate renal dysfunction (creatinine: 1.21 mg/dL) and slightly elevated levels of serum carcinoembryonic antigen and serum soluble interleukin 2 receptor (6.1 ng/mL and 635 IU/mL, respectively). Other laboratory data were within normal limits.

Imaging examinations

A contrast-enhanced abdominal CT scan revealed two irregular and highly contrast-enhanced masses at the neck and body of the gallbladder as well as periportal lymph node enlargement, measuring 30 mm × 20 mm in diameter and consistent with gallbladder cancer lymph node metastasis (Figure 1). Magnetic resonance imaging revealed that the tumor signal was hypointense on T1-weighted imaging and hyperintense on T2-weighted and diffusion-weighted imaging (Figure 2). Positron emission tomography (PET) revealed increased ^{18}F-fluorodeoxyglucose uptake in the tumor (Figure 3). Endoscopic ultrasonography (EUS) showed a heterogeneous echoic mass extending into the lumen, but it did not infiltrate the serosa (Figure 4).

FINAL DIAGNOSIS

The final diagnosis was gallbladder adenocarcinoma with lymph node metastasis.

TREATMENT

On the basis of these findings, the patient underwent bile duct resection with gallbladder bed resection and periportal lymph node dissection. The surgical findings revealed the gallbladder tumor was relatively softer than ordinary gallbladder cancers. The swollen lymph node was firm but did not invade the portal vein. However, the patient developed severe aspiration pneumonia and bile leakage after the surgery, which were treated conservatively. The patient was discharged 2 mo after the surgery.

OUTCOME AND FOLLOW-UP

Histologic examination revealed periportal lymphadenopathy and two tumors at the neck and body of the gallbladder, measuring 27 mm × 20 mm and 20 mm × 18 mm in diameter, respectively. Histological findings also showed monotonous lymphoid cells with hemophagocytosis by macrophages. Immunohistochemical staining for markers showed the presence of CD10, BCL6, and c-Myc and the absence of BCL2. The Ki-67 index was > 80% (Figure 5). Therefore, the patient was finally diagnosed as having Burkitt's lymphoma. Although the surgical margin was pathologically negative, recurrence was noted at the hepatic radical margin and superior pancreaticoduodenal lymph nodes on PET-CT immediately after discharge. Thus, he was referred to a hematologist and started receiving treatment with reduced-dose cyclophosphamide, doxorubicin, vincristine, and prednisone.

DISCUSSION

Malignant lymphoma of the gallbladder is a rare form of gallbladder malignancy, which accounts for 0.1%-0.2% of all gallbladder cancers[1-3]. In previous reports, most of these malignancies were documented to be diffuse large B-cell lymphomas and MALTomas[2,4], and only two reports had documented Burkitt's lymphoma of the gallbladder[6,7]. Compared to previous cases, the present case yielded some interesting clinical and imaging findings.

Table 1 Diagnostic criteria for gastrointestinal malignant lymphoma

Ref.	Diagnostic criteria
Dawson *et al*[8]	Absence of palpable superficial lymphadenopathy
	Absence of obvious enlargement of mediastinal lymph nodes
	Normal level of total and differential white blood cell counts
	The bowel lesion predominating and the only lymph node obviously affected being those in its immediate neighborhood
	Absence of tumor in the liver and spleen
Lewin *et al*[9]	Exhibiting gastroinetstinal symptoms or predominant lesions in the gastrointestinal tract

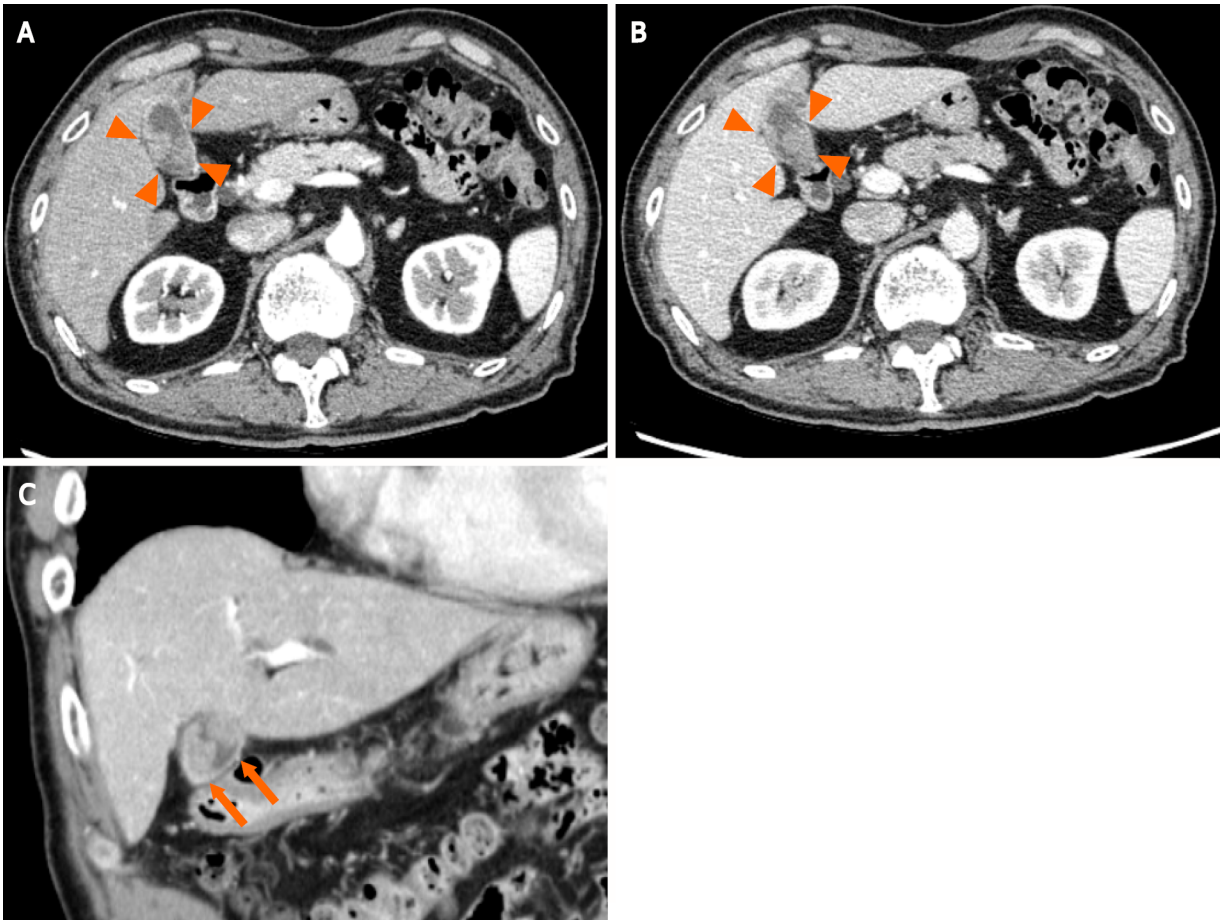


Figure 1 A contrast-enhanced abdominal computed tomography scan shows two irregular and highly contrast-enhanced masses (arrowheads and arrow) at the neck and body of the gallbladder as well as periportal lymph node enlargement, which is consistent with gallbladder cancer lymph node metastasis. A: Axial section image in the early phase showing neck of the gallbladder; B: Axial section image in the delayed phase showing neck of the gallbladder in the delayed phase; C: Coronal sectional image showing body of the gallbladder.

First, this is potentially the first reported case of "primary" gallbladder Burkitt's lymphoma. According to the diagnostic criteria of primary gastrointestinal lymphoma (Table 1) defined by Dawson *et al*[8] and Lewin *et al*[9], the previous two cases were diagnosed as "secondary" gallbladder Burkitt's lymphoma because they included extra-gallbladder lesions, such as duodenal, hepatic, or central nervous system lesions. In contrast, in the present case, the tumor was localized in the gallbladder and a periportal lymph node. Although no gastrointestinal symptoms were observed because the primary site was the gallbladder, other criteria were fulfilled. Therefore, the patient was diagnosed as having "primary" gallbladder Burkitt's lymphoma, and the case was considered novel.

Second, gallbladder Burkitt's lymphoma can present as a localized disease that mimics gallbladder cancer. The imaging features of malignant lymphoma were reported by Ono *et al*[3], who reported that high-grade malignant lymphomas showed

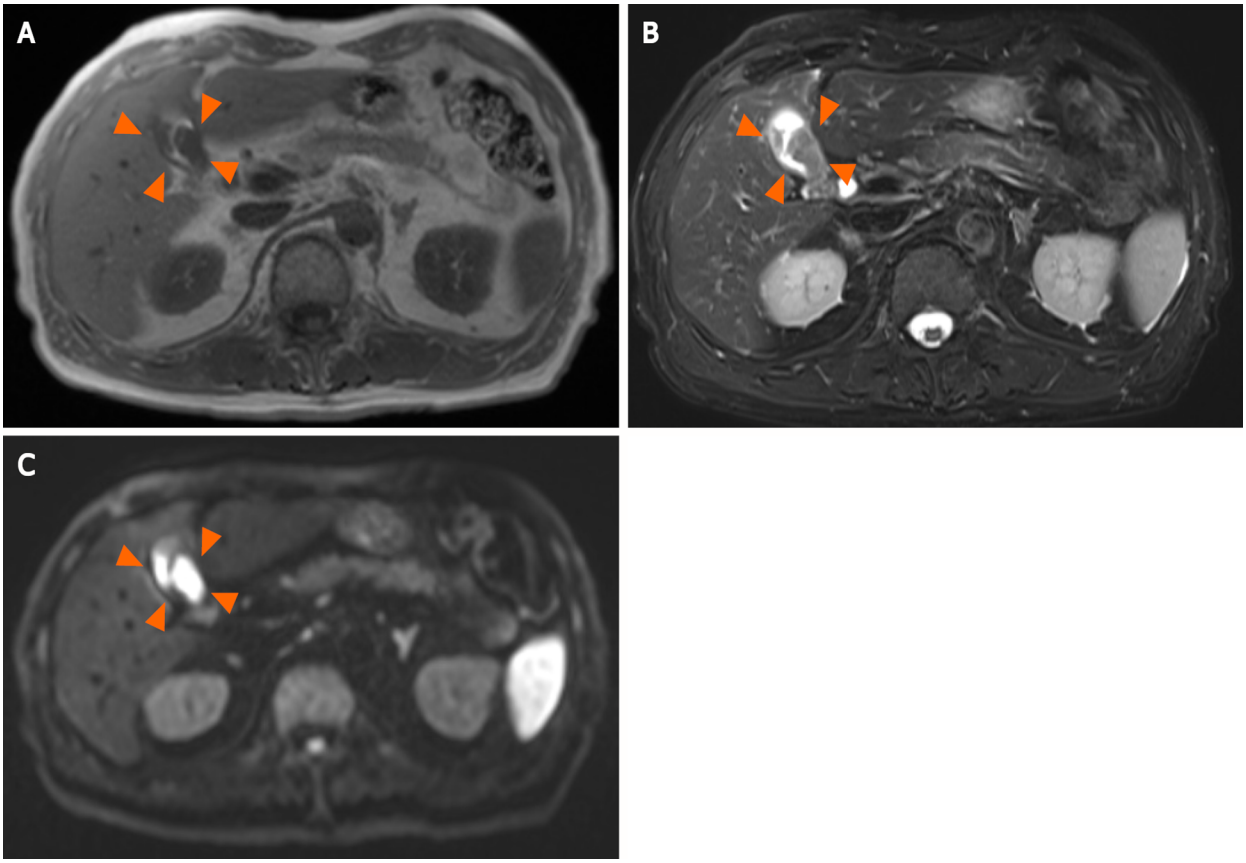


Figure 2 Magnetic resonance imaging reveals a hypointense tumor signal. A: T1-weighted imaging (arrowheads); B: A hyperintense signal on T2-weighted imaging (arrowheads); C: Diffusion-weighted imaging (arrowheads).

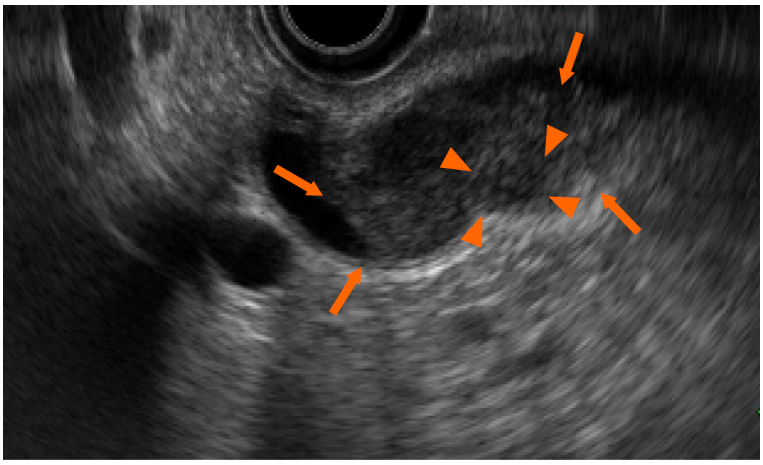


Figure 3 Endoscopic ultrasonography shows a heterogeneous echoic mass (arrows) with internal partially low echo (arrowheads). The mass extends into the lumen but does not infiltrate the serosa.

solid and bulky masses or irregular wall thickening. In addition, because Burkitt's lymphoma is a highly aggressive and rapidly progressive disease, some extranodal sites are generally involved at the time of diagnosis[10] and tumor localization around the primary lesion is rare[11]. In this case, the malignancy was localized in the body of the gallbladder and a periportal lymph node. Since these findings were consistent with the imaging findings of gallbladder adenocarcinoma, we could not confirm a preoperative diagnosis of malignant lymphoma.

Unfortunately, the patient had lymphoma recurrence 2 mo after the surgery because the introduction of chemotherapy had to be delayed owing to postoperative complications. Burkitt's lymphoma is a highly aggressive disease, but it is highly sensitive to chemotherapy. Therefore, chemotherapy has the highest priority in the treatment of

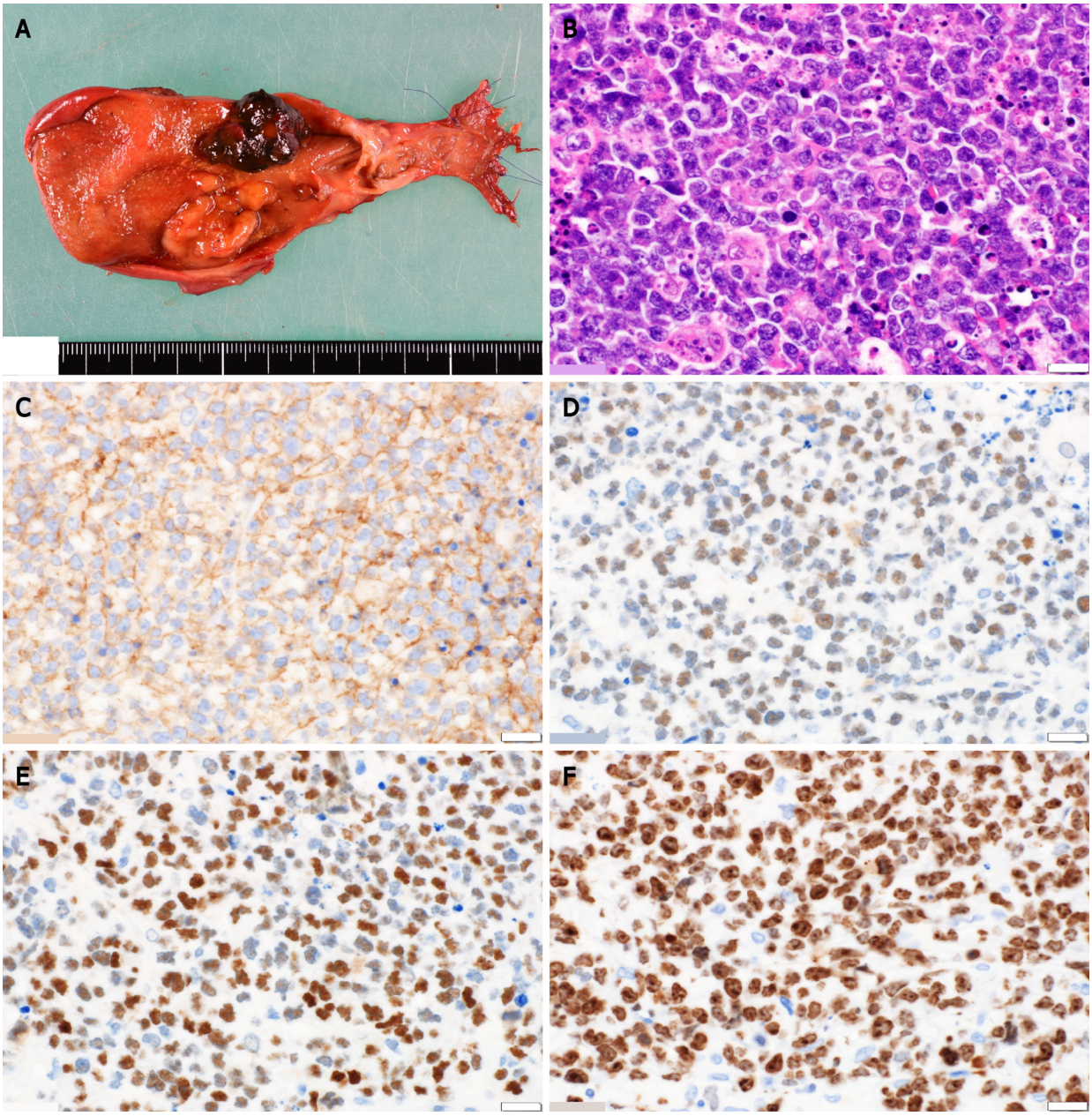


Figure 4 Histologic examination. A: Periportal lymphadenopathy and two tumors at the neck and body of the gallbladder, measuring 27 mm × 20 mm and 20 mm × 18 mm in diameter, respectively; B: Histological findings reveal monotonous lymphoid cells with hemophagocytosis by macrophages; C-E: Immunohistochemical staining for markers shows the presence of CD10 (C), BCL6 (D), and c-Myc (E) and the absence of BCL2; F: The Ki-67 index is > 80%. The white scale bars represent 1 mm.

Burkitt's lymphoma[10], and we should have introduced chemotherapy as soon as possible after the surgery. If we had been aware of the possibility of gallbladder malignant lymphoma, we could have avoided the extended procedure and could have initiated chemotherapy at the appropriate time.

Although the accurate preoperative diagnosis of gallbladder malignant lymphoma is quite difficult[2,12], some previous reports[3,12,13] have suggested the possibility of a precise preoperative diagnosis based on imaging findings. Specifically, Ono *et al*[3] showed that the signal intensity of the gallbladder wall on T2-weighted imaging is more hypointense in malignant lymphoma than in carcinoma. In addition, Kato *et al*[12] reported the usefulness of an internal partially low echo in distinguishing malignant lymphoma from carcinoma. On a retrospective review of the present case, although no obvious differences were observed in the intensity of T2-weighted imaging when compared to that of gallbladder cancers, EUS revealed an internal partially low echo of the tumor. Furthermore, in this case, a discrepancy existed in that no serosal invasion was observed despite the size of the tumor and presence of lymphadenopathy. We think this might be a characteristic finding that distinguishes

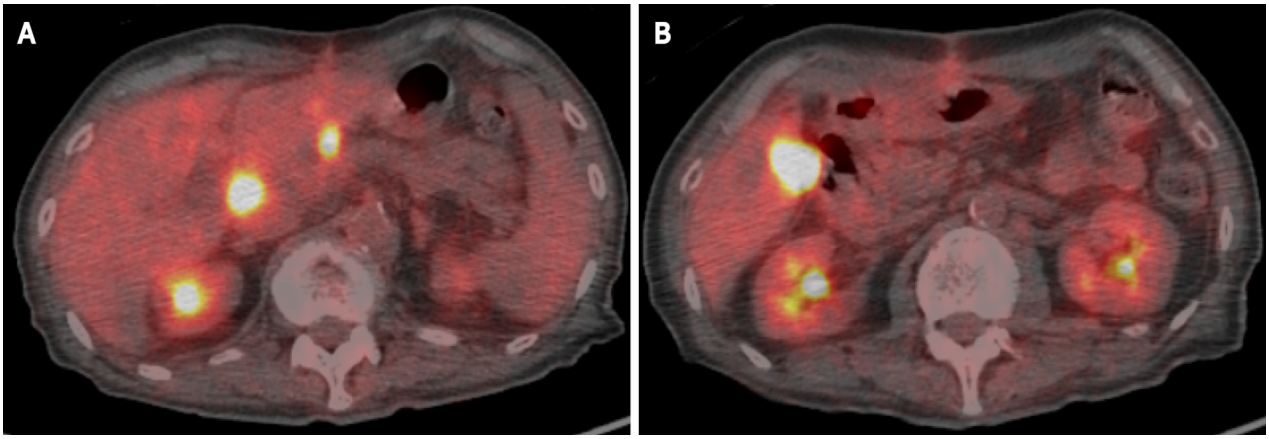


Figure 5 Positron emission tomography reveals increased ^{18}F-fluorodeoxyglucose uptake at the superior pancreaticoduodenal lymph nodes and hepatic radical margin. A: Superior pancreaticoduodenal lymph nodes; B: Hepatic radical margin.

gallbladder malignant lymphoma from carcinoma.

Nevertheless, a biopsy examination seems the best diagnostic technique in the present case, considering the possibility of malignant lymphoma, and hence, biopsy should be planned for patients with the above imaging findings.

CONCLUSION

Burkitt's lymphoma can occur in the gallbladder. Therefore, this disease should be considered in the differential diagnosis of a gallbladder tumor, and biopsy can be useful in facilitating the early introduction of chemotherapy in cases with suggestive imaging findings.

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LETTER TO THE EDITOR

COVID-19, liver dysfunction and pathophysiology: A conceptual discussion

Jucier Gonçalves Júnior

ORCID number: Jucier Gonçalves Júnior 0000-0001-5077-7959.

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Jucier Gonçalves Júnior, Department of Internal Medicine, Division of Rheumatology, São Paulo University, São Paulo 01246-903, State, Brazil

Corresponding author: Jucier Gonçalves Júnior, MD, PhD, Academic Research, Department of Internal Medicine, Division of Rheumatology, São Paulo University, Av. Dr. Arnaldo, 455, 3º Andar-Sala 3131 Cerqueira César, São Paulo 01246-903, State, Brazil.
juciergjunior@hotmail.com

Abstract

The intra and extracellular pathways of hepatic injury by coronavirus disease 2019 (COVID-19) are still being studied. Understanding them is important to treat this viral disease and other liver and biliary tract disorders. Thus, this paper aims to present three hypotheses about liver injury caused by COVID-19: (1) The interactions between severe acute respiratory syndrome coronavirus 2 spike protein and membrane receptors in the hepatocyte; (2) The dysbiosis and "gut-liver axis" disruption in patients with serious clinical presentations of COVID-19; and (3) The inflammatory response exacerbated through the production of interleukins such as interleukin-6. However, despite these new perspectives, the pathophysiological process of liver injury caused by COVID-19 is still complex and multifactorial. Thus, understanding all these variables is a challenge to science but also the key to propose individualized and effective patient therapies.

Key Words: COVID-19; Intracellular signaling peptides and proteins; Immunopathology; Liver diseases; Liver injury; SARS-CoV-2

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Core Tip: This paper aimed to present new hypotheses on the pathophysiology of liver injury caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Interactions between SARS-CoV-2 spike protein and other membrane receptors in the liver; "gut-liver axis" disruption and dysbiosis; and increased inflammatory process mediated by interleukin-6 and AT1R-metalloprotease 17 seem to be factors that contribute to such injury.

Citation: Gonçalves Júnior J. COVID-19, liver dysfunction and pathophysiology: A conceptual

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TO THE EDITOR

I have read the work of Prof. Gracia-Ramos *et al*[1] about the clinical aspects of the relationship between liver dysfunction and coronavirus disease 2019 (COVID-19). The author aimed to summarize the pathophysiology, clinical importance, and management of COVID-19 in patients with or without preexisting liver disease.

I would like to highlight some hypotheses for the pathophysiological impairment of the liver in COVID-19. To facilitate visualization, I have summarized the findings in Figure 1. I believe the information provided will enrich the current discussion and may enhance the results of the aforementioned paper[1].

The first theory states that liver cells have two receptors that have an affinity with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein. The first receptor is the Cluster of Differentiation 147 (CD147) or basigin (BSG) or Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)[2], and the second receptor is the Liver/Lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin (L-SIGN)[3].

CD147 is a transmembrane glycoprotein of the immunoglobulin superfamily overexpressed in an inflammatory process triggered by viral infections (*e.g.*, Severe Acute Respiratory Syndrome in 2002), bacterial infections, and parasitic infections (*e.g.*, Plasmodium falciparum)[2] (Figure 1). Evidence of CD147 protein expression in the liver tissue was found in 1999[4]. Recently, a United States publication in Nature journal highlighted the possibility that a chimeric anti-CD147 receptor would be a possible treatment for hepatocellular carcinoma[5]. Experimental research has shown affinity between CD147 and SARS-CoV-2 spike protein. A Chinese study published in Nature journal evaluating the *in vitro* association between the CD147 receptor and the SARS-CoV-2 spike protein by enzyme-linked immunosorbent assay and plasmon resonance demonstrated an affinity of 1.85 × 10–7 Michaelis between them. In parallel, the authors demonstrated that in cell cultures, when the CD147 protein is blocked by specific autoantibodies (*e.g.*, meplazumab), SARS-CoV-2 amplification is inhibited. Additionally, the virus was able to enter into naturally non-susceptible cells (*e.g.*, cells of baby hamster lineage) more easily when CD147 expression was induced in this population[6]. Moreover, increased expression of CD147 in tissues outside the lung has also been shown as an alternative pathway for SARS-CoV-2 infection in bioinformatics studies[7] and systematic reviews[8]. Therefore, an interesting hypothesis would be that the affinity between the CD147 receptor and the SARS-CoV-2 spike protein represents another way for the virus to infect liver cells.

L-SIGN is a liver-specific membrane receptor related to viral capture[3]. L-SIGN is already widely studied in diseases that affect the liver, such as diseases caused by the hepatitis C virus, the human immunodeficiency virus, the Rift Valley fever virus, the Uukuniemi virus, and the Toscana virus[9]. A recent study supports this hypothesis by suggesting that L-SIGN may provide a new way for SARS-CoV-2 to enter human cells [10]. In COVID-19, autopsy studies showed that SARS-CoV-2-infected hepatic sinusoid cells expressed more L-SIGN receptors compared to control groups[11]. Besides that, the literature has shown *in vitro* interactions between L-SIGN and the spike protein[12, 13], in which this receptor binds to angiotensin II (ACE2), increasing the capacity of SARS-CoV-2 to infect liver cells[8,14] (Figure 1).

The second hypothesis (Figure 1) highlights the apparent "gut–liver axis disruption"[15] caused by COVID-19. More than half (60%) of the patients infected with SARS-CoV-2 developed liver injury[16], and some studies have already shown that the hepatic clearance of toxins is negatively impacted by COVID-19[17]. This shows there are varying degrees of dysfunction. Past infections by H1N1[15] and SARS-like viruses[18] led to severe cytopathic alterations in the gastrointestinal tract within 48 h of the beginning of the infectious process[19]. Thus, the disruption of this cross-talk would lead to two consequences: (1) Bacterial translocation stimulating septic shock; and (2) Perpetuation of the septic shock that would lead to a worsened ischemic state[15,20].

The literature has shown a decrease in commensal bacteria and pathogenic microorganisms in patients with COVID-19. This situation persists even after the absence of symptoms and an undetectable viral load by reverse transcription-polymerase chain

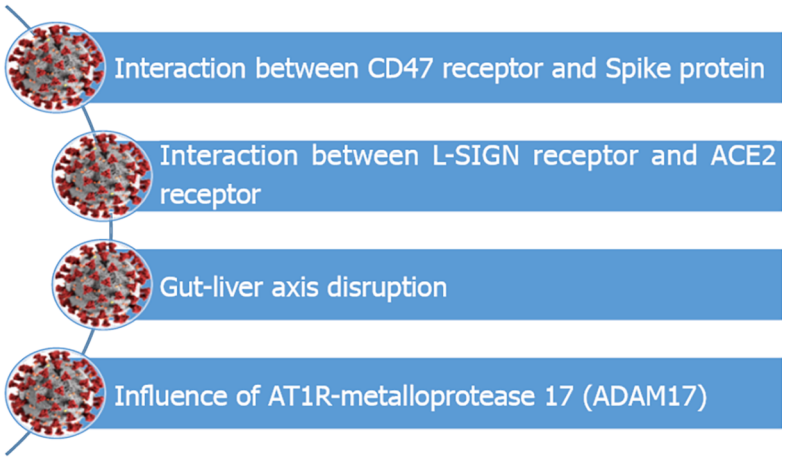


Figure 1 Pathophysiological hypotheses explaining liver injury by severe acute respiratory syndrome coronavirus-2. ACE2: Angiotensin II; ADAM17: AT1R-metalloprotease 17; CD147: Cluster of Differentiation 147; L-SIGN: Liver/Lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin.

reaction. Increased colony-forming units of opportunistic bacteria, such as Veillonella spp., Rothia spp., Actinomyces spp.[20], Faecalibacterium prausnitzii, Clostridium ramosum, and Clostridium hathewayi, in the fecal sample of patients with COVID-19, have been associated with more severe illness by SARS-CoV-2[19]. Furthermore, subspecies of Bacteroides sp. (which decrease ACE2 expression in murine intestine models), when present in human fecal samples, have been correlated with a lower viral load of SARS-CoV-2[21,22]. Besides that, epidemiological studies[23] and meta-analyses[24] have shown that COVID-19 can cause cellular dysfunction in enterocytes. More than half (54%) of the patients infected with COVID-19 had SARS-CoV-2 RNA in their fecal samples in a Chinese study[25]. A paper published by Mazza *et al*[26] demonstrated the presence of fecal calprotectin in a patient infected with COVID-19 showing direct damage to the gastric mucosa. Thus, the disruption of the gastric mucosa feeds back the "cytokine storm" caused by COVID-19 and can lead to hepatic tissue injury[27].

Parohan *et al*[28], when analyzing 3428 patients with COVID-19, demonstrated a significant increase in serum aspartate aminotransferase, alanine aminotransferase, and total bilirubin levels with lower levels of albumin in critically ill patients. An epidemiological survey showed that 62% of the patients admitted to intensive care units (ICUs) had increased liver enzymes. Furthermore, in these ICUs, patients had higher values of pro-inflammatory cytokines such as interleukins (IL) 10, 7, 2; monocyte chemoattractant protein-1 (MCP1); gamma induced protein 10 (IP-10); granulocyte colony-stimulating factor (GCSF); and tumor necrosis factor α (TNF-α) when compared to their controls not admitted to ICUs[29]. Indeed, autopsy studies of patients with severe acute respiratory syndrome caused by COVID-19 showed centrilobular sinusoidal dilation and lobular infiltration by small lymphocytes[30]. Percutaneous liver biopsy of patients infected with coronavirus showed histopathological findings suggestive of liver injury, such as acidophilic bodies, hepatocyte ballooning, and lobular activity without fibrin deposition or fibrosis[16].

The third theory is that SARS-CoV-2 endocytosis by immune system cells is caused by AT1R-metalloprotease 17 (ADAM17), which is also involved in the genesis of liver injury (Figure 1). The mechanism by which ADAM17 facilitates viral entry is not yet known. However, it is known that the increase in its activity can lead to the cleavage of pro-inflammatory molecules (*e.g.*, IL-6; TNF-α), reinforcing the inflammatory process and injury to various organs, including the liver, during SARS-CoV-2 infection[31,32]. Additionally, ADAM17 breaks down several proteins that are responsible for liver regeneration/protection. Among ADAM17 substrates are the epidermal growth factor receptor (EGFR) ligand amphiregulin (AR), the heparin-binding-EGF-like growth factor (HB-EGF), and the hepatocyte growth factor (HGF). ADAM17 deletion in cell cultures of hepatocytes led to a decrease in EGFR and HB-EGF (responsible for preventing liver injury). These molecules increased the apoptosis of hepatocytes and decreased their proliferation[33].

Interestingly, studies have shown increased serum levels of ADAMS17 in comorbidities known to be risk factors for severe cases of COVID-19, such as heart failure[34], COPD[35], diabetes mellitus[36], kidney disease[37], and increasing age

[34]. On the other hand, decreased ADAM17 activity is correlated with decreased ACE2 receptors, thus having a protective effect against SARS-CoV-2 infections[38].

Therefore, the pathophysiological process of liver injury caused by COVID-19 is complex, multifactorial, and extensive. There are many (intra and extracellular) inflammatory pathways we are not yet aware of, in addition to local and systemic environmental factors that interfere. Understanding all these variables is a challenge to science. Additionally, only with this understanding, we will be able to propose individualized and effective therapies.

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LETTER TO THE EDITOR

Comments on validation of conventional non-invasive fibrosis scoring systems in patients with metabolic associated fatty liver disease

Jian-Guo Hong, Lun-Jie Yan, Xian Li, Sheng-Yu Yao, Peng Su, Hai-Chao Li, Zi-Niu Ding, Dong-Xu Wang, Zhao-Ru Dong, Tao Li

ORCID number: Jian-Guo Hong 0000-0003-1529-1106; Lun-Jie Yan 0000-0002-1102-1144; Xian Li 0000-0001-7347-8628; Sheng-Yu Yao 0000-0002-6911-0465; Peng Su 0000-0003-1495-2689; Hai-Chao Li 0000-0002-4220-1295; Zi-Niu Ding 0000-0002-2308-8629; Dong-Xu Wang 0000-0002-4550-4611; Zhao-Ru Dong 0000-0003-2906-0210; Tao Li 0000-0002-5378-3059.

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Jian-Guo Hong, Lun-Jie Yan, Xian Li, Sheng-Yu Yao, Hai-Chao Li, Zi-Niu Ding, Dong-Xu Wang, Zhao-Ru Dong, Tao Li, Department of General Surgery, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Peng Su, Department of Pathology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Corresponding author: Tao Li, MD, Chief Doctor, Department of General Surgery, Qilu Hospital of Shandong University, No. 107 West Wenhua Road, Jinan 250012, Shandong Province, China. litao7709@126.com

Abstract

To evaluate and predict liver fibrosis in patients with nonalcoholic fatty liver disease (NAFLD), several non-invasive scoring systems were built and widely used in the progress of diagnosis and treatment, which showed great diagnostic efficiency, such as aspartate aminotransferase to platelet ratio index, fibrosis-4 index, body mass index, aspartate aminotransferase to alanine aminotransferase ratio, diabetes score and NAFLD fibrosis score. Since the new concept of metabolic associated fatty liver disease (MAFLD) was proposed, the clinical application value of the non-invasive scoring systems mentioned above has not been assessed in MAFLD. The evaluation of the diagnostic performance of these non-invasive scoring systems will provide references for clinicians in the diagnosis of MAFLD.

Key Words: Metabolic associated fatty liver disease; Prediction model; Calibration; Normal distribution; Nonalcoholic fatty liver disease

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Core Tip: The concept of metabolic associated fatty liver disease (MAFLD) was proposed in 2020. Unlike the concept of nonalcoholic fatty liver disease, the exclusion of chronic liver disease was not required in the establishment of diagnosis of MAFLD,

quality classification
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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but the presence of metabolic associated disease or dysfunction is required. The clinical prediction values and the optimal cutoff values of non-invasive fibrosis scores remain unknown. We read the recent article entitled "Validation of Conventional Non-invasive Fibrosis Scoring Systems in Patients with Metabolic Associated Fatty Liver Disease" with great interest. We would like to share our opinions and criticisms about this valuable work.

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TO THE EDITOR

We read the recent article entitled "Validation of Conventional Non-invasive Fibrosis Scoring Systems in Patients with Metabolic Associated Fatty Liver Disease" published by Wu *et al*[1] with great interest. In the article, the authors designed and performed a retrospective study to evaluate the diagnostic performance of four non-invasive scoring systems, including aspartate aminotransferase to platelet ratio index (APRI), fibrosis-4 index (FIB-4), body mass index (BMI), aspartate aminotransferase to alanine aminotransferase (ALT) ratio, diabetes score (BARD score) and nonalcoholic fatty liver disease fibrosis score (NFS), in patients with metabolic associated fatty liver disease (MAFLD). We would like to share our opinions and criticisms about this valuable work.

The specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and discrimination of the scoring systems mentioned above were evaluated in the prediction of advanced fibrosis in patients with MAFLD in the study by Wu *et al*[1]. Clinical characteristics, laboratory variables and non-invasive scores were compared between patients with advanced fibrosis and those with mild fibrosis or without fibrosis. The results showed that the FIB-4 ($P < 0.001$), NFS ($P < 0.001$), APRI ($P = 0.003$) and BARD ($P < 0.001$) scores were all significantly higher in patients with advanced fibrosis. Unfortunately, only univariate analysis was performed in this study. In our opinion, multivariate analysis should be performed to recognize the independent variables in the prediction of advanced fibrosis, as in the study by Nielsen *et al*[2].

The authors evaluated the diagnostic efficiency of the prediction scores using the following statistical indices: Sensitivity, specificity, accuracy, PPV, NPV and the area under the receiver operating characteristic curve (AUROC). All the above indices are important statistical variables in the development and validation of prediction models. In our opinion, it would be better if the authors had evaluated the calibration of the prediction scores in the study. In fact, calibration of the prediction model is a critical statistical index in the evaluation of diagnostic efficiency[3]. Calibration of the prediction scores can be performed using the Hosmer–Lemeshow goodness-of-fit test and calibration curves, the latter of which can be easily plotted using R software. We advise the authors to evaluate the calibration of prediction models in future studies.

The calculations of PPV and NPV are very important in the development and validation of prediction models. Unlike sensitivity and specificity, PPV and NPV cannot be compared directly among different samples, except for samples with the same prevalence rate of the disease. This is because both PPV and NPV can be affected by the prevalence rate of disease[4,5]. The authors compared the PPV and NPV in table 4 and stated that "PPV and NPV was better in the HBV-MAFLD group" in the article. In our opinion, the comparisons of PPV and NPV between the HBV-MAFLD group and the pure MAFLD group will be valuable only when advanced fibrosis accounts for the same proportion of the two groups.

Although the authors stated that the continuous variables were expressed in the format of mean ± SD or median value with interquartile range (IQR) and the differences were calculated using Student's *t* test in the case of normally distributed data or the Mann–Whitney test in the remaining cases, there were no continuous variables expressed as the median (IQR) in the article. Normally, the distribution of

continuous variables should be tested; then, the continuous variables in normal distribution will be expressed as the mean ± SD, and the non-normally distributed continuous variables will be expressed as the median (IQR). If all the continuous variables are expressed as the mean ± SD but the authors do not indicate that all the continuous variables fit a normal distribution, the readers will doubt whether the normal distribution tests were performed in the study. After all, it rarely happens that all the laboratory variables fit a normal distribution in one study. In most studies, the laboratory variables and scores, including ALT, AST, APRI and other variables, do not fit a normal distribution and should be expressed as the median (interquartile range) [2,6-8]. For any parameter in biomedical research, a true normal distribution is rare. The European Medicines Agency has issued the general guidance that data should be checked for normality of distribution and should be analyzed and presented based on the results of normal distribution tests. It is also possible that some continuous variables did not fit a normal distribution, but these variables were accidentally expressed as the mean ± SD in the study conducted by Wu *et al*[1]. Of course, this situation indeed does not affect the accuracy of the study. We advise that the authors indicate whether the continuous variables fit a normal distribution and if the normal distribution tests have been performed in future studies.

The authors compared the diagnostic ability of the NFS, FIB-4, APRI and BARD score for a late stage of fibrosis in MAFLD. The results demonstrated that the APRI and BARD scores performed poorly, but the FIB-4 and NFS showed a promising prospect in clinical use. The new thresholds of the FIB-4 and NFS proposed in this study were 1.05 and -2.1, respectively. The two thresholds proposed by the authors were determined based on their specific study sample. The diagnostic efficiency of the thresholds in the prediction of advanced fibrosis should be further evaluated in an external validation cohort and/or in a prospective validation cohort. Additionally, if possible, the authors can try to develop models based on multiple variables, including the FIB-4 and/or NFS, to predict advanced fibrosis in patients with MAFLD. He *et al*[9] proposed that a diagnostic model containing valuable parameters extracted from more examination tools might provide more satisfactory results[9]. Compared to using a single variable, we believe that prediction models based on multiple variables, including clinical characteristics, radiology examinations and laboratory examinations, would exhibit higher sensitivity, higher specificity, higher accuracy, higher PPV, higher NPV, better discrimination and better calibration in the prediction of advanced fibrosis in patients with MAFLD. Because there is now evidence from a prospective cohort that common genetic variants can capture additional prognostic insights not conveyed by validated clinical/biochemical parameters[10], we encourage the integration of genetics (perhaps epigenetics) with clinical fibrosis scores, as it may refine individual risk and improve risk stratification and prediction of severe liver disease.

In general, we are very interested in the study by Wu *et al*[1]. The authors demonstrated the prediction values of APRI, FIB-4, NFS and BARD in a large sample of histology-proven MAFLD. As MAFLD is a new entity, this study will provide important references for clinicians in the prediction of advanced fibrosis in MAFLD patients. The study performed by Wu *et al*[1] could also provide important references for other studies of non-invasive scores and prediction models.

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Contents

Weekly Volume 28 Number 7 February 21, 2022

EDITORIAL

693 Early gastric cancer: A challenge in Western countries

Chiarello MM, Fico V, Pepe G, Tropeano G, Adams NJ, Altieri G, Brisinda G

MINIREVIEWS

704 Will the collaboration of surgery and external radiotherapy open new avenues for hepatocellular carcinoma with portal vein thrombosis?

Choe JW, Lee HY, Rim CH

ORIGINAL ARTICLE

Retrospective Study

715 Clinical online nomogram for predicting prognosis in recurrent hepatolithiasis after biliary surgery: A multicenter, retrospective study

Pu T, Chen JM, Li ZH, Jiang D, Guo Q, Li AQ, Cai M, Chen ZX, Xie K, Zhao YJ, Wang C, Hou H, Lu Z, Geng XP, Liu FB

Observational Study

732 Effect of *Bifidobacterium longum* 35624 on disease severity and quality of life in patients with irritable bowel syndrome

Sabaté JM, Iglicki F

SYSTEMATIC REVIEWS

745 Stereotactic radiotherapy and the potential role of magnetic resonance-guided adaptive techniques for pancreatic cancer

Ermongkonchai T, Khor R, Muralidharan V, Tebbutt N, Lim K, Kutaiba N, Ng SP

CASE REPORT

755 Crohn's disease-related 'gastrocnemius myalgia syndrome' successfully treated with infliximab: A case report

Catherine J, Kadhim H, Lambot F, Liefferinckx C, Meurant V, Otero Sanchez L

LETTER TO THE EDITOR

763 Gallbladder biliary lithotripsy: A new rationale applied to old treatment

Dioscoridi L, Mutignani M

766 Gastrointestinal microbiome and *Helicobacter pylori*: Eradicate, leave it as it is, or take a personalized benefit–risk approach?

Sitkin S, Lazebnik L, Avalueva E, Kononova S, Vakhitov T

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Weekly Volume 28 Number 7 February 21, 2022

Contents

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Editorial Board Member of *World Journal of Gastroenterology*, Madhusudana Girija Sanal, MBBS, PhD, Academic Fellow, Assistant Professor, Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Scieneces, New Delhi 110070, Delhi, India. sanalmg@gmail.com

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EDITORIAL

Early gastric cancer: A challenge in Western countries

Maria Michela Chiarello, Valeria Fico, Gilda Pepe, Giuseppe Tropeano, Neill James Adams, Gaia Altieri, Giuseppe Brisinda

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Maria Michela Chiarello, Department of Surgery, Azienda Sanitaria Provinciale di Crotone, Ospedale San Giovanni di Dio, Crotone 88900, Italy

Valeria Fico, Gilda Pepe, Giuseppe Tropeano, Gaia Altieri, Emergency Surgery and Trauma Center, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome 00168, Italy

Neill James Adams, Health Sciences, Clinical Microbiology Unit, Magna Grecia University, Catanzaro 88100, Italy

Giuseppe Brisinda, Department of Medical and Surgical Sciences, Catholic School of Medicine, Rome 00168, Italy

Giuseppe Brisinda, Department of Surgery, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome 00168, Italy

Corresponding author: Giuseppe Brisinda, MD, Department of Surgery, Fondazione Policlinico Universitario A Gemelli IRCCS, Largo A Gemelli 8, Rome 00168, Italy. gbrisin@tin.it

Abstract

Early gastric cancer (EGC) is an invasive carcinoma involving only the stomach mucosa or submucosa, independently of lymph node status. EGC represents over 50% of cases in Japan and in South Korea, whereas it accounts only for approximately 20% of all newly diagnosed gastric cancers in Western countries. The main classification systems of EGC are the Vienna histopathologic classification and the Paris endoscopic classification of polypoid and non-polypoid lesions. A careful endoscopic assessment is fundamental to establish the best treatment of EGC. Generally, EGCs are curable if the lesion is completely removed by endoscopic resection or surgery. Some types of EGC can be resected endoscopically; for others the most appropriate treatment is surgical resection and D2 lymphadenectomy, especially in Western countries. The favorable oncological prognosis, the extended lymphadenectomy and the reconstruction of the intestinal continuity that excludes the duodenum make the prophylactic cholecystectomy mandatory to avoid the onset of biliary complications.

Key Words: Early gastric cancer; Diagnosis; Treatment; Endoscopic resection; Surgery; Lymph nodes metastases

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Core Tip: Early gastric cancer (EGC) is an invasive stomach cancer confined to the mucosal or submucosal lining and represents approximately 20% of gastric cancers in Western countries. A correct classification allows the most appropriate treatment. Some types of EGC are adequately treated by endoscopic mucosal resection, whilst others need gastrectomy. In Western countries, due to a higher incidence of the diffuse histotype and the less widespread advanced endoscopic procedures, surgical resection and D2 lymphadenectomy are regarded as the "gold standard" treatment.

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INTRODUCTION

In its initial stages, gastric cancer tends to spread in the lamina propria, infiltrating the muscularis mucosae and the submucosal layer of the gastric wall[1-4]. The Japanese Society for Gastroenterological Endoscopy's definition of early gastric cancer (EGC) is an invasive gastric cancer that invades no more deeply than the submucosa, irrespective of lymph node metastases (LNM). Thanks to rigorous screening programs in the Asian countries, up to 50% of patients treated for a gastric cancer have EGC [5,6]. Such a high incidence is not found in European countries, where advanced gastric cancer is prevalent.

The difference in incidence between Asian and Western countries may be due to a wide use of magnifying upper endoscopy with high-resolution images and chromoendoscopy in Japan and South Korea.

A correct classification and an accurate diagnosis are fundamental to plan the most effective treatment[7-10]. Some forms of EGC can be treated by endoscopic resection; in other forms surgical treatment is mandatory[10-14].

EGC carries an excellent prognosis if the lesion is completely removed by endoscopic resection or surgery[15-17]. Most of the Japanese studies have reported 5-year and 10-year survival rates of more than 90% for the patients with EGC. In the Western studies, 5-year survival rates are variable, ranging from 68% to 92%. EGCs recur in at least 1.9% of cases after resection with time intervals ranging from 4 mo to more than 10 years. Important risk factors for the recurrence are the presence of submucosal invasion, LNMs and undifferentiated histology.

CLASSIFICATION

The most common classification is the TNM, which relates information about the primitive lesion (T), node involvement (N) and distant metastases (M) thus providing information about the diffusion of the disease.

The macroscopic classification of EGC, as defined by the Japanese Gastric Cancer Association (JGCA), identifies different subtypes: type 0-I (protruding-polypoid tumors); type 0-IIa (superficial elevated tumors); type 0-IIb (tumors without elevation or depression); type 0-IIc (slightly depressed tumors); and type 0-III (excavated tumors), referring to the morphological features of the lesion on the mucosal surface[18].

The Vienna classification (Table 1) is a histologic classification. While the TNM defines EGC as a cancer invading no more deeply than the submucosa, irrespective of LNMs, the Vienna classification is the most exhaustive[19,20]. In particular, the intramucosal EGC is classified in 3 categories, while the submucosal EGC in 2, according to the depth of infiltration. This distinction is not present in the TNM system and is fundamental for the treatment planning. In fact, it allows the option for an endoscopic therapy [(endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD)] or surgery if mandatory.

The Paris classification of polypoid and non-polypoid lesions including flat lesions is useful to evaluate possible endoscopic treatments. This classification was created for the superficial colon neoplasms, undoubtedly the most frequent in the Western World and with some small changes was adopted to the superficial gastric neoplasms. One of the changes is the thickness of the wall layer, as in the submucosa, are thinner in the stomach.

Table 1 Vienna classification

Category	Definition	Treatment
1	Negative for dysplasia/neoplasia	No treatment
2	Indefinite for dysplasia/neoplasia	Follow-up, recheck
3	Non-invasive, low-grade dysplasia	Follow-up
4	Non-invasive, high-grade dysplasia	Endoscopic resection, surgery
4.1	High-grade adenoma/dysplasia	
4.2	Non-invasive carcinoma (CIS)	
4.3	Suspicious of invasive carcinoma	
5	Invasive neoplasia	Surgery (recently ESD)
5.1	Intramucosal carcinoma	
M1	Mucosa only	
M2	Mucosa with preservation of muscularis mucosae	
M3	Not further than muscularis mucosae	
5.2	Submucosal carcinoma or beyond	
sm1	Invasion of muscularis mucosae < 0.5 μm	
sm2	Invasion of muscularis mucosae > 0.5 μm	

CIS: Cancer *in situ*; ESD: Endoscopic submucosal dissection.

DIAGNOSIS

A correct endoscopic diagnosis is fundamental in EGC[21]. The Japanese Society for Gastroenterological Endoscopy suggests that the length of the endoscopy, the accurate stomach wall distension and the further removal of mucous from the stomach lining are proportional to diagnostic accuracy and lesion detection rate[22]. As far as the length of the procedure is concerned, the most appropriate protocol is the Systematic Screening Stomach, which requires careful vision of the gastric lumen and acquisition of 22 pictures (12 in anterograde vision and 10 in retro vision) with a global length of the exam not inferior to 12 min[22]. Sedation is advised, while trans-nasal endoscopy is not.

Regarding diagnostic accuracy, the use of traditional or virtual cromo-endoscopy is advised to look for the "minimal changes" of the Japanese authors to evaluate the size and morphology of the lesion and the glandular and vascular pattern. Lesions should be described according to the Paris classification (Type 0I protruding, Type 0-IIa elevated, Type 0-IIb flat, Type 0-IIc depressed, Type 0-III excavated)[23].

TREATMENT

Endoscopic resection must be taken into account when the risk of LNMs is low or when the location and size of the tumor allows a safe "en-bloc" complete resection[24,25]. Selected EGCs can be treated with EMR or ESD with good results in Western patients[26-28]. Absolute indications for endoscopic resection, according to the Japanese gastric cancer treatment guidelines, are: cT1a differentiated-type adenocarcinoma without ulcerative findings or cT1a differentiated-type adenocarcinoma with ulcerative findings and a diameter 3 cm. Endoscopic resection for cT1a undifferentiated carcinoma without ulcerative findings and with a diameter 2 cm is considered an expanded indication. Therefore, endoscopic resection should be considered for elderly patients with a high operative risk (relative indication)[18,29].

According to JGCA guidelines (Table 2), en-bloc endoscopic resection for differentiated pT1a cancer without ulcerative findings (UL0), negative horizontal margin (HM0), negative vertical margin (VM0) and no lymphovascular infiltration (Ly0, V0) or en-bloc endoscopic resection for pT1a cancer with ulcerative findings, tumor size 3 cm, HM0, VM0, Ly0 and V0 are classified as endoscopic curability A. However, the resection is classified as endoscopic curability B for undifferentiated pT1a cancer resected en-bloc, tumor size 2 cm, without ulcerative findings, HM0, VM0, Ly0 and V0 or for en-bloc resected pT1b differentiated cancer, with a depth of infiltration < 500 μm, tumor size 3 cm, HM0, VM0, Ly0 and V0. When the resection fulfills criteria to be classified as endoscopic curability A or endoscopic curability B, but the cancer was not resected en-bloc or has positive horizontal margin, it is classified as

Table 2 Criteria for endoscopic mucosal resection, endoscopic submucosal dissection and surgery

	Mucosal cancer				Submucosal cancer	
	No ulcer		Ulcer present		SM1	SM2
Size	≤ 20	> 20	≤ 30	> 30	≤ 30	Any size
Differentiated	Y	G	G	R	G	R
Undifferentiated	Gray	R	R	R	R	R

Y: Yellow is guideline criteria for endoscopic mucosal resection; G: Green is guideline criteria for endoscopic submucosal dissection; R: Red is surgery; Gray: Expanded indication for endoscopic submucosal dissection; SM1: Submucosal invasion < 500 μm; SM2: Submucosal invasion ≥ 500 μm.

endoscopic curability C-1. All other resections are classified as endoscopic curability C-2.

Both endoscopic curability A and endoscopic curability B are considered to have low risk of LNMs and do not require additional surgery, so the patient can go to follow-up. Endoscopic curability C-1 has a low risk of LNMs but should be aware of residual or local recurrence, so the patient could be treated with another ESD, surgical resection, close observation or endoscopic coagulation. After an endoscopic resection classified as endoscopic curability C-2, gastrectomy with lymphadenectomy should be considered the standard-of-care[18].

The results of endoscopic treatment (EMR and ESD) have been compared to traditional surgical treatment in patients with EGC in several meta-analyses[30]. Overall survival after EMR or ESD is not significantly different when compared to surgical resection (hazard ratio: 0.995, 95% confidence interval: 0.836-1.185; $P = 0.9$). As for recurrence free survival, it has been proved that the recurrence risk after EMR is significantly higher compared to surgical resection (hazard ratio: 3,946, 95% confidence interval: 1.233-12.632, $P = 0.02$)[30]. However, it has been noted that in adequately selected patients endoscopic resection offers results that are similar to those of traditional surgery[31]. In patients who underwent EMR/ESD, strict endoscopic surveillance is mandatory, given the higher risk of endoluminal recurrence.

Complications of EMR or ESD for EGC include pain, bleeding and perforation[32]. Pain after resection is typically mild. Bleeding occurs in up to 8% of patients undergoing endoscopic treatment. Immediate bleeding appears more common with EMR/ESD of tumors located in the upper third of the stomach. Perforation occurs in 4% of the patients during ESD[15].

An innovative procedure called laparoscopic endoscopic cooperative surgery (LECS) that combines the strongest points of interventional endoscopy and laparoscopic surgery for the removal of gastric wall tumors was developed by Hiki *et al*[33]. In the original procedure of LECS, the stomach wall is opened for resection of the tumor, and the lumen is exposed to intraperitoneal space. LECS involves precutting around the tumor with an endoscope and artificial perforation of the gastric wall. Next, excision of the tumor with laparoscopy and repair of the gastric wall with a stapler are performed[34, 35].

The indications for LECS are currently limited to lesions that are normally managed by ESD but are technically challenging to resect *via* ESD because of the presence of ulcers, or if the tumor size is more than 30 mm in diameter[36].

When a LECS procedure and sentinel node biopsy were combined, an extremely minimally invasive procedure that is adequate for radical oncological resection of EGC is achieved. Sentinel node navigation surgery is an ideal surgical option for preservation of most parts of the stomach and consequent maintenance of normal gastric function to improve quality of life in patients with EGC. Although many previous studies and clinical trials have demonstrated the safety and feasibility of the sentinel node concept in gastric cancer, the clinical application of sentinel node navigation surgery is debatable. Several issues regarding technical standardization and oncological safety need to be resolved. Recently several studies to resolve these problems are being actively performed, and sentinel node navigation surgery might be an important surgical option in the treatment of gastric cancer in the future.

Other techniques (Table 3) have been developed to avoid spread of the tumor to the peritoneum[37-39]. When performed by expert teams they show a lot of promise and achieve solid oncologic results[40, 41]. Use and standardization of these minimally invasive surgical procedures contributes to reduction of unnecessary gastrectomy for gastric submucosal tumors[42]. Most of the clinical experiences are Japanese, and the small number of treated cases does not allow a comparison with the longest used endoscopic and surgical techniques. More research and clinical trials about LECS for EGC are expected. Furthermore, surgeons need to select one of these minimally invasive procedures according to the characteristics of the tumor, the localization of the tumor in the stomach, the personal surgical experience and the technological characteristics of the health institution.

In the case of EGC without the above features, total or partial gastrectomy with a free margin of at least 2 cm is the treatment of choice. Adequate lymphadenectomy offers a high chance of curing patients

Table 3 Laparoscopic endoscopic cooperative procedures

Procedure	Characteristics
Laparoscopic endoscopic cooperative surgery	Endoscopic dissection of the mucosal or submucosal layers with laparoscopic seromuscular resection.
Endoscope-assisted laparoscopic wedge resection	The procedure is performed to remove tumors with a laparoscope after localization by an intraoperative endoscope. EAWR is difficult to implement in sites where strictures may occur, such as pylorus and the gastroesophageal junction.
Laparoscopy-assisted endoscopic resection	The concept of LAER is contrary to that of EAWR. The procedure is an ESD procedure assisted by laparoscopy.
Endoscope-assisted laparoscopic transgastric resection	The procedure involves opening of the gastric wall under the direct view of an endoscope, tagging the tumor with a laparoscopic suture and performing wedge resection with a laparoscopic stapler.
Laparoscopic intragastric surgery	Procedure can be used in laparoscopic surgery performed within the stomach. The incision in the wall of the stomach is minimized and laparoscopic trocars are inserted into the gastric lumen.
Single-incision intragastric resection	This is a single-port laparoscopic surgery.
Endoscopic submucosal dissection with laparoscopic lymph node dissection	This procedure is the same as LAER with laparoscopic perigastric lymph node dissection. The advantage is that the stomach can be preserved. However, the main procedure is ESD, which requires a skilled endoscopist.
Single-incision endoscopic submucosa dissection with laparoscopic lymph node dissection	The procedure is similar to SI-IGR, where sentinel node navigation surgery with unilateral perigastric laparoscopic lymph node dissection is performed with a single-port. Then ESD is performed through a single-port.
Laparoscopy-assisted endoscopic full-thickness resection	If the tumor invades deeper than the muscle layer of the wall of the stomach, full-thickness resection with an endoscope is performed and a laparoscope is used for repair.
Non-exposed wall-inversion surgery	The procedure was developed so that EFTR could be performed without spillage. The disadvantages are that the procedure time is long, as it involves ESD and endoscopic closure, and it is difficult to apply to the pyloric area and gastroesophageal junction.
Clean no-exposure technique	Similar to NEWS, this procedure has also been developed to avoid cancer cell spillage. Clean-NET can be applied to EGCs in most locations, except for pyloric area and gastroesophageal junction.

EAWR: Endoscope-assisted laparoscopic wedge resection; LAER: Laparoscopy-assisted endoscopic resection; ESD: Endoscopic submucosal dissection; EATR: Endoscope-assisted laparoscopic transgastric resection; SI-IGR: Single-incision intragastric resection; EFTR: Endoscopic full thickness resection; NEWS: Non-exposed wall-inversion surgery; Clean-NET: Clean no-exposure technique; EGC: Early gastric cancer.

with EGC[43-45]. The lymph nodes of the stomach are classified into stations numbered as in Table 4. The regional stations are the lymph node stations 1-12 and LN station 14v (Figure 1A). The remnant stations are considered as distant stations, and metastases to these nodes are classified as M1. The JGCA defined the extent of systematic lymphadenectomy according to the type (total or distal) of gastrectomy indicated (Table 5).

Surgical treatment is also mandatory when there is a lack of training in complex endoscopic procedures. In hospitals with a low volume of advanced endoscopic procedures, gastrectomy remains the gold standard for treatment of EGC.

In EGC lymphatic invasion is early and is linked to the distribution of the lymphatic vessels in the mucosa and submucosa, which form a rich plexus located just above, inside and below the muscularis mucosae. Infiltration of the mucosa (EGC T1a) is sufficient to cause LNMs. Submucosal infiltration (EGC T1b) entails a higher incidence of LNMs. In addition, the risk is higher in cases where the submucosal invasion has destroyed the muscularis mucosae (5.3% of intramucosal cancer and in 19.6%-64.3% of submucosal neoplasms)[46,47].

As already known, tumor grading and vascular-lymphatic invasion were found to be significant risk factors. A study on 517 patients with EGC found that LNMs are present in 22% of cases (114 patients), directly related to an ulcerated lesion of more than 2 cm diameter. Furthermore, the risk of LNMs is higher in males (P = 0.03), elderly patients (P = 0.01), in the presence of depressed tumor (P = 0.01) and with submucosal invasion (P = 0.03). Further risk factors include tumor localization in the body (P = 0.04) or at the angulus (P = 0.02). In these areas, the submucosa is thinner, and the lymphatic vessels more widespread in the lamina propria of the mucosa.

Similar results have been documented in a recent paper, where age, gender, tumor size, type of differentiation, Lauren classification and lympho-vascular and perineural invasion showed a significant correlation with the rate of LNMs in EGC by univariate and multivariate analyses in 1033 patients who underwent radical gastrectomy with lymphadenectomy. Patients with T1b gastric cancer had an older age, a higher proportion of proximal lesions, larger tumor size, more frequent vascular lymphatic invasion, perineural invasion and more LNMs than patients with T1a gastric cancer[48-51].

In a series of 5265 Japanese patients that underwent gastrectomy and lymphadenectomy, 3016 lesions were intramucosal EGCs, and 2249 were infiltrating the submucosa. LNMs were present in 65 (2.2%) of the patients with intramucosal EGC and in 402 (17.9%) of those with penetrating EGC. The authors

Table 4 Anatomical definitions of lymph node stations

Nr.	Definition
1	Right paracardial LNs, including those along the first branch of the ascending limb of the left gastric artery
2	Left paracardial LNs including those along the esophagocardiac branch of the left subphrenic artery
3	3a: Lesser curvature LNs along the branches of the left gastric artery; 3b: Lesser curvature LNs along the 2nd branch and distal part of the right gastric artery
4	(1) 4sa: Left greater curvature LNs along the short gastric arteries (perigastric area); (2) 4sb: Left greater curvature LNs along the left gastroepiploic artery (perigastric area); and (3) 4d: Right greater curvature LNs along the 2nd branch and distal part of the right gastroepiploic artery
5	Suprapyloric LNs along the 1st branch and proximal part of the right gastric artery
6	Infrapyloric LNs along the first branch and proximal part of the right gastroepiploic artery down to the confluence of the right gastroepiploic vein and the anterior superior pancreatoduodenal vein
7	LNs along the trunk of left gastric artery between its root and the origin of its ascending branch
8	8a: Anterosuperior LNs along the common hepatic artery; 8p: Posterior LNs along the common hepatic artery
9	Coeliac artery
10	Splenic hilar LNs including those adjacent to the splenic artery distal to the pancreatic tail, and those on the roots of the short gastric arteries and those along the left gastroepiploic artery proximal to its 1st gastric branch
11	(1) 11p: Proximal splenic artery LNs from its origin to halfway between its origin and the pancreatic tail end; and (2) 11d: Distal splenic artery LNs from halfway between its origin and the pancreatic tail end to the end of the pancreatic tail
12	(1) 12a: Hepatoduodenal ligament LNs along the proper hepatic artery, in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas; (2) 12b: Hepatoduodenal ligament LNs along the bile duct, in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas; and (3) 12p: Hepatoduodenal ligament LNs along the portal vein in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas
13	LNs on the posterior surface of the pancreatic head cranial to the duodenal papilla
14	LNs along the superior mesenteric vein
15	LNs along the middle colic vessels
16	(1) 16a1: Paraaortic LNs in the diaphragmatic aortic hiatus; (2) 16a2: Paraaortic LNs between the upper margin of the origin of the celiac artery and the lower border of the left renal vein; (3) 16b1: Paraaortic LNs between the lower border of the left renal vein and the upper border of the origin of the inferior mesenteric artery; and (4) 16b2: Paraaortic LNs between the upper border of the origin of the inferior mesenteric artery and the aortic bifurcation
17	LNs on the anterior surface of the pancreatic head beneath the pancreatic sheath
18	LNs along the inferior border of the pancreatic body
19	Infradiaphragmatic LNs predominantly along the subphrenic artery
20	Paraesophageal LNs in the diaphragmatic esophageal hiatus

LNs: Lymph nodes.

Table 5 Extent of systematic lymphadenectomy according to the type (total or distal) of gastrectomy indicated

Lymphadenectomy	Gastrectomy	
	Total	Distal
D1	Lymph node stations from N. 1 to 7	Lymph node stations N. 1, 3, 4sb, 4d, 5, 6 and 7
D1+	D1 stations plus stations N. 8a, 9 and 11p	D1 stations plus stations N. 8a and 9
D2	D1 stations plus stations N. 8a, 9, 10, 11p, 11d and 12a	D1 stations plus stations N. 8a, 9, 11p and 12a.

documented that in the intramucosal cancer, the risk of LNMs is higher in depressed or ulcerated lesions, in lesions bigger than 30 mm, in those with an undifferentiated type or in those with vascular and lymphatic invasion. In submucosal EGCs a higher incidence of LNMs was documented in submucosal invasion ≥ 500 μm (23.7%) compared to submucosal invasion < 500 μm (8.8%, $P < 0.0001$), when there was vascular lymphatic invasion (36.6% *vs* 9.8% if no infiltration, $P < 0.0001$), in case of elevated EGC (24.8%) compared to depressed type (17.1%, $P = 0.0003$) and in tumors larger than 30 mm ($P < 0.0001$). Moreover, extra-gastric LNMs (level II) were found in 10% of the cases, in the absence of involvement of the peri-gastric lymph nodes.

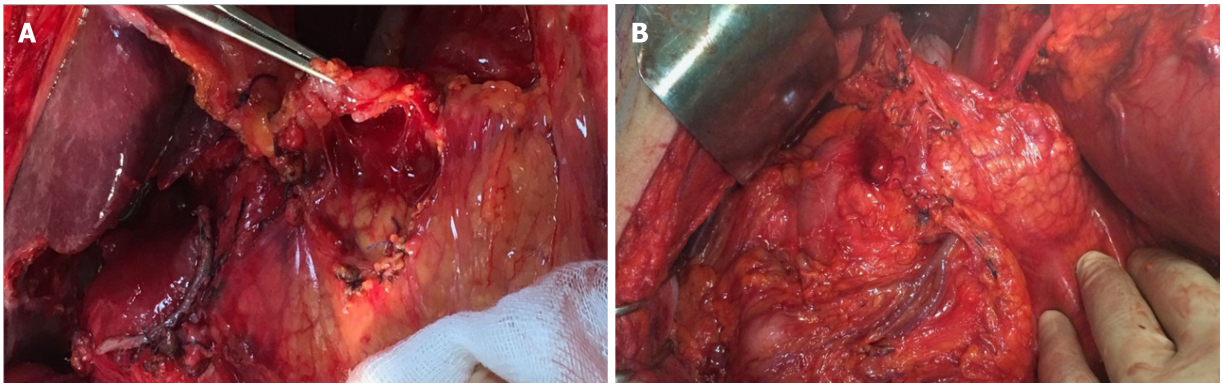


Figure 1 Lymph node removal along the upper border of the pancreas (lymph node station N. 12a) (A), along the common hepatic artery (lymph node station N. 8a) and along the left gastric artery (lymph node station N. 9) (B). Personal experience: Consecutive patients were recruited by senior surgeons (MMC, GB).

To optimize lymphadenectomy, the sentinel node search has been proposed. Said procedure is now standardized in breast cancer surgery but still experimental in the case of EGC[52,53]. Two endoscopically administered tracers are used sequentially: the 99Tc radioisotope (at least 3 h before the intervention) and the blue isosulphan dye (during the intervention). Since skip LNMs are frequent (about 20% of cases in EGCs), a negative sentinel node does not exclude the possibility that subsequent lymph node stations are involved in the disease. The usefulness of the sentinel lymph node would therefore not consist in excluding LNMs, as initially proposed, but in identifying the lymphatic drainage basin that must be removed. Thus, the sentinel lymph node would therefore become an aid to identify the relationship between gastric anatomy and gastric lymphatic drainage.

LNM strongly affects the prognosis[14,54]. This is a topic of not negligible importance because EGC is believed to be a curable albeit malignant disease. In T1a EGCs, the 10-year survival rates are about 100% in patients undergoing gastric resection and extended (D2) lymphadenectomy[55]. Five-year and ten-year survival in these patients was 2%–3% higher than in those with limited (D1) lymphadenectomy. In the case of submucosal EGC, the 10-year survival rate was about 100% in patients undergoing gastric resection and extended lymphadenectomy. Five-year and ten-year survival was 10% higher in these patients than in those with limited lymphadenectomy. Looking at the postoperative lymph node positive (pN+) patient group, 10-year survival was 87% if extended lymphadenectomy was performed, and 55% if lymphadenectomy was limited.

In patients with a radical resection, when a D2 lymphadenectomy (Figure 1B) was performed and the duodenum was excluded in the intestinal reconstruction, cholecy-stectomy, considered by some to be a non-essential measure, is necessary to avoid gallstone formation and its complications.

The use of 300 mg or 600 mg of ursodeoxycholic acid compared with placebo resulted in a significantly decreased proportion of patients developing gallstones within 12 mo after surgery for gastric cancer (5.3% in the 300 mg group, 4.3% in the 600 mg group and 16.7% in the placebo group) [56]. Gallstone formation and its complications may be prevented using ursodeoxycholic acid rather than prophylactic cholecystectomy. However, the results raise some questions. As Pitt and Nakeeb[57] maintained, the study was conducted in patients undergoing gastrectomy for stomach cancer but follow-up did not last more than a year. Furthermore, more than 80% of patients have EGCs with a presumptively satisfactory oncological prognosis, given that 84% of cancers occurred in the distal stomach and 57% of lymphadenectomies were D2[56]. In this setting, we believe that prophylactic cholecystectomy is necessary for patients with a good cancer prognosis, as suggested by Pitt and Nakeeb[57].

Studies on the subject conclude that prophylactic cholecystectomy does not have a significant impact on the natural course of the disease[58]. However, it leads to a reduction in the number of biliary complications (which may affect up to 15% of the operated patients) and does not induce an increase in mortality and morbidity rates. In one study, a mortality rate of 1.8% was reported in the case of cholecystectomy performed during an intervention other than that of the gastric cancer treatment. Prophylactic cholecystectomy seems to be unnecessary only in cases where the continuity of the digestive tract involves the use of the duodenum[59]. In a study on 969 patients, after gastrectomy, gallbladder stones developed in 6.1% of patients (59 out of 969) with an incidence of cholecystitis /cholangitis of 1.2% of cases (12/969). It has been found that the method used to restore intestinal continuity, with preservation of the duodenal transit or excluding the duodenum, is an independent risk factor for both the development of cholelithiasis (P = 0.018) and cholecystitis and cholangitis (P = 0.006). It has been also confirmed that in patients who develop cholelithiasis, the incidence of cholecystitis and cholangitis is particularly high when the duodenal transit was excluded (31.3%) compared to those with duodenal transit maintained (7.4%).

CONCLUSION

In agreement with the JGCA treatment guidelines, D1 lymphadenectomy can be sufficient to treat EGCs not suitable for endoscopic treatment when the regional nodes are clinically free from disease. It is important to point out, however, that a relevant percentage of EGCs in Western countries are of the diffuse type and therefore associated with a high risk of LNMs, especially when the submucosa is invaded. Moreover, in Western countries, endoscopic resections are not very common, and preoperative diagnosis of lymph node status has some limitations, despite advancements in radiological techniques. Considering these limitations, D2 lymphadenectomy in EGCs not susceptible to radical endoscopic treatment is the surgical treatment of choice. In this area, based on a good prognosis and intestinal reconstruction that excludes the duodenum from gastrointestinal transit, we believe that prophylactic cholecystectomy is necessary.

FOOTNOTES

Author contributions: Chiarello MM and Brisinda G conceived the original idea; Fico V, Pepe G, Altieri G and Tropeano G performed a comprehensive review of all available literature and synthesized the data; Chiarello MM, Fico V, Adams NJ and Brisinda G wrote the manuscript; Chiarello MM, Fico V, Pepe G, Altieri G, Tropeano G and Brisinda G contributed to the study design, manuscript structure and performed a final critical appraisal of the manuscript; Chiarello MM, Fico V, Pepe G, Tropeano G, Adams NJ, Altieri G and Brisinda G read and approved the final manuscript.

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Country/Territory of origin: Italy

ORCID number: Maria Michela Chiarello 0000-0003-3455-0062; Valeria Fico 0000-0003-1619-4164; Gilda Pepe 0000-0001-9852-6243; Giuseppe Tropeano 0000-0001-9006-5040; Neill James Adams 0000-0002-2813-8648; Gaia Altieri 0000-0002-0324-2430; Giuseppe Brisinda 0000-0001-8820-9471.

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MINIREVIEWS

Will the collaboration of surgery and external radiotherapy open new avenues for hepatocellular carcinoma with portal vein thrombosis?

Jung Wan Choe, Hye Yoon Lee, Chai Hong Rim

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Jung Wan Choe, Department ofInternal Medicine, Korea University Ansan Hospital, Ansan 15355, South Korea

Hye Yoon Lee, Department of Surgery, Korea University Ansan Hospital, Ansan 15355, South Korea

Chai Hong Rim, Department of Radiation Oncology, Korea University Ansan Hospital, Ansan 15355, South Korea

Corresponding author: Chai Hong Rim, MD, PhD, Assistant Professor, Department of Radiation Oncology, Korea University Ansan Hospital, 123 Jeokgeum-ro, Danwon-gu, Ansan 15355, South Korea. crusion3@naver.com

Abstract

Portal invasion of hepatocellular carcinoma (HCC) occurs in 12.5%-40% of patients diagnosed with cancer and yields poor clinical outcomes. Since it is a common cause of inoperability, sorafenib was regarded as the standard treatment for HCC in the Barcelona Clinic of Liver Cancer guidelines. However, the median survival of the Asian population was only approximately 6 mo, and the tumor response rate was less than moderate (< 5%). Various locoregional modalities were performed, including external beam radiotherapy (EBRT), transarterial chemoembolization, hepatic arterial infusion chemotherapy, and surgery, alone or in combination. Among them, EBRT is a noninvasive method and can safely treat tumors involving the major vessels. Palliative EBRT has been commonly performed, especially in East Asian countries, where locally invasive HCC is highly prevalent. Although surgery is not commonly indicated, pioneering studies have demonstrated encouraging results in recent decades. Furthermore, the combination of neo- or adjuvant EBRT and surgery has been recently used and has significantly improved the outcomes of HCC patients, as reported in a few randomized studies. Regarding systemic modality, a combination of novel immunotherapy and vascular endothelial growth factor inhibitor showed results superior to that of sorafenib as a first-line agent. Future clinical trials investigating the combined use of these novel agents, surgery, and EBRT are expected to improve the prognosis of HCC with portal invasion.

Key Words: Surgery; Hepatocellular carcinoma; Radiotherapy; Systemic treatment

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Core Tip: The prognosis of hepatocellular carcinoma with portal vein involvement is poor, and there had been few available local modalities. However, with the develop-ment of radiotherapy techniques, the 1-year survival rate has been reported to be close to 45%-50% after palliation. Recently, a surgical approach has also been attempted showing encouraging results. Furthermore, the combination of surgery and radiation therapy showed effective results in studies including randomized studies. The combination of these two modalities are expected to increase efficacy of treating hepatocellular carcinoma with portal invasion.

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INTRODUCTION

Portal invasion of hepatocellular carcinoma (HCC) is a known clinical factor associated with poor prognosis. Portal vein invasion might cause portal hypertension, which can lead to decreased liver function and enlarge the gastrointestinal varices[1]. In addition, portal tumor thrombosis acts as a tumor deposit that can induce intra- and extrahepatic metastases[2,3]. Without active treatment, the survival period of HCC patients with portal invasion is usually less than 4 mo[4-6]. Portal invasion occurs in 12.5%-40% of all HCC patients in the clinical setting[7-11]. According to the 16th National Survey for Primary Liver Cancer conducted in Japan, portal involvement was found in 16% (808/5130) of patients who underwent hepatic resection[12]. In our previous study using data from the Nationwide Liver Cancer Registry, 2553 of 10743 patients (approximately 10% randomly extracted patient records from all administrative districts of South Korea), or about a quarter, had portal vein involvement at the time of diagnosis[13].

To date, there is no standard treatment for HCC with portal invasion; hence, various systemic and locoregional modalities have been used. Since portal invasion is a component of the Barcelona Clinic of Liver Cancer (BCLC) stage C, the European Association for the Study of the Liver (EASL)[14] and American Association for the Study of Liver Diseases (AASLD) guidelines[15], which were published in 2018, support the use of sorafenib for the treatment of HCC with this stage. Although sorafenib was the only modality that achieved an overall survival (OS) benefit, as reported in a phase 3 randomized study conducted within a period of 15 years, the tumor response rate was not satisfactory (less than 5%), and the median OS was only 6 mo after treatment as shown in an Asian population study[16]. In addition, it should not be overlooked that approximately 95% of the enrolled patients were in Child-Pugh class A in this study, even though a significant portion of the HCC patients with PVT have a liver function of Child-Pugh class B or C[17]; data on the use of sorafenib in these patients is limited. A randomized phase 3 trial was done comparing sorafenib *vs* best supportive care alone in Child-Pugh class B patients, and the results are expected to provide further guidance[18]. A recent study investigated the use of regorafenib for the HCC patients who have failed to improve on sorafenib; the median OS of 10.6 mo was achieved in patients who received regorafenib, which was better than the OS of 7.8 mo for those receiving the placebo[19]. Although several studies on the expansion of indications for systemic and rescue therapies are in progress, satisfactory results have not yet been obtained.

Therefore, various locoregional modalities have been used to improve the patient outcomes. Despite that the AASLD guidelines endorsed treatment recommendations of BCLC system previously[20], in its updated version in 2018, it is mentioned that systemic therapy for HCC with macrovascular invasion is recommended but not over locoregional treatments[15]. The preferred option of locoregional modality was not suggested, although recent studies regarding transarterial chemoembolization (TACE), external beam radiotherapy (EBRT), and hepatic arterial infusion chemotherapy (HAIC) were referenced, because evidence is limited to suggest relative superiority among those modalities.

The application of locoregional treatment for HCC with portal invasion varies regionally, and treatment strategies also differ between Eastern and Western countries[21]. In Asian countries, hepatitis B virus-related HCCs comprise the majority of diagnosed diseases. These diseases are mainly caused by insertional mutagenesis of viral oncoproteins and tend to progress rapidly and invasively, whereas liver function can be relatively preserved. In Western countries, the common causes of HCC include alcohol consumption or fatty liver of other causes. These diseases are associated with somatic alterations and chromosomal instability accumulated due to cirrhosis progression; they tend to be less progressive and have higher differentiation, but the liver function is commonly damaged due to persistent cirrhosis[22]. Therefore, the active locoregional treatments including TACE, surgery, EBRT, and HAIC are more rigorously attempted in Asia than in Western countries[21]. For instance, the surgical approach is preferentially recommended for portal invasion cases that do not invade the main branch, as indicated

in the recent Chinese expert consensus guidelines[23], although the vast majority of international guidelines did not suggest surgery as the preferred option for those cases[24,25]. Based on the Korean Liver Cancer Study Group guidelines, the combination of TACE and EBRT was suggested as the best option for HCC patients without extrahepatic metastases but with major vessel invasion[26].

Various locoregional modalities have been used independently or in combination to treat HCC with portal invasion. In particular, despite the fact that surgery is not generally recommended for portal invasive cases, recent studies have achieved favorable oncologic results by reducing the extent of tumors through EBRT and surgery. In this review, we aimed to discuss the role of locoregional modalities in the treatment of HCC with portal invasion, focusing on the recent results of surgery and EBRT as individual treatments and as a combination therapy.

PALLIATING PORTAL INVASION WITH EXTERNAL RADIOTHERAPY

In the early 1990s, before the generalization of a planning system using computed tomography, EBRT was known to have a limited role in HCC. EBRT had been "technically radioresistant" during the era of two-dimensional radiotherapy, of which the treatment was planned and performed under plain radiographic guidance. Since EBRT relies on bony and organ shadows available on plain films, radiation therapy inevitably encompasses a significant portion of the normal liver. Due to the possibility of hepatic radiotoxicity, only doses lower than 30 Gy could be administered, which is not sufficient to control the tumor[24,27].

Although the risk of radiation-induced liver disease steeply increased after delivering 30 Gy of irradiation to the entire liver, CT-based planning has enabled the safe delivery of high doses of over 50 Gy. The application of a more updated technology, such as intensity-modulated radiotherapy (a special type of EBRT that increases the conformality of the radiation target by actively modulating the dose intensity, portal shape, and beam movements), enabled the irradiation of advanced intrahepatic tumors while saving the necessary portion of the normal liver (Figure 1). Furthermore, despite the fact that tumor vascular invasion makes the application of local modalities difficult, EBRT is feasible because the major blood vessels can withstand more than approximately 100 Gy (in conventional fraction) and the target dose can be delivered regardless of the location of tumor[28]. Indeed, the HCC cells are radiosensitive and highly proliferative tumor cells with an alpha/beta ratio of approximately 15, which is similar to that of head and neck cancer (a radiosensitive cancer commonly treated with chemotherapy or radiotherapy)[29,30]. Previous studies using radiation therapy for locally advanced HCCs, which recruited patients from the 90s to the 2000s, have shown that HCC responds well to high doses of radiation. In a previous study including 158 unresectable HCC patients (about 50% of patients had portal invasion), Park *et al*[31] reported that the tumor response rates in patients who received irradiation doses of < 40 Gy, 40-50 Gy, or > 50 Gy increased to 29.2%, 68.6%, and 77.1%, respectively. Similarly, our previous study reported that as a result of prescribing a median total EBRT dose of 61.2 Gy, a high tumor response rate of 62.2% was reported in 45 HCC patients with portal vein thrombosis (PVT)[32].

Various studies have reported the use of EBRT in HCC patients with portal invasion in recent decades. In order to integrate the data from several studies, we performed a meta-analysis of 26 studies and 2,111 HCC patients treated with EBRT[33]. The pooled 1- and 2-year OS rates were 43.8% [95% confidence interval (CI): 37.6%–50.2%] and 22.3% (17.7%-27.6%) in patients who underwent three-dimensional conformal radiotherapy (3DCRT), and 48.5% (39.4%-57.8%) and 26.8% (19.0%-36.3%) in patients who underwent stereotactic body radiotherapy (SBRT). The pooled tumor response rates were 51.5% (95%CI: 45.7%-57.0%) and 70.7% (95%CI: 63.7%-76.8%) in patients who underwent 3DCRT and SBRT, respectively. Severe gastrointestinal or hepatic complications rarely occurred and were either not reported in most studies or were documented in less than 5%-10% of treated patients. Various locoregional modalities have been applied for the treatment of unresectable HCCs, and TACE is the most common modality performed in combination with EBRT. Huo *et al*[34] reported that the combination of TACE and RT had higher survival benefit [odds ratio (OR): 1.36, 95%CI: 1.19-1.54 for 1-year OS] and complete response rate (OR: 2.73, 95%CI: 1.95-3.81) than TACE alone for unresectable HCCs. They also pointed out that the benefits of OS progressively increased (ORs: 1.55, 1.91, 3.01, and 3.98 for 2-, 3-, 4-, and 5-year OS, respectively). In a recent randomized trial, Yoon *et al*[35] reported that the combination of TACE and EBRT improved the OS (median: 55 wk *vs* 43 wk, $P = 0.04$) and progression-free survival (median: 31 wk *vs* 11.7 wk, $P < 0.001$) of HCC patients with major vascular invasion compared with sorafenib. Recently, our team performed a study using nationwide data from all administrative districts of South Korea[13]. Strict propensity matching was performed, and data of 444 HCC patients with portal invasion (222 who underwent local treatment including EBRT and 222 who did not undergo active oncologic treatment) were analyzed. Local treatment, including radiotherapy, had significant benefits on the OS (median: 8 mo *vs* 2 mo, $P < 0.001$) and cancer-specific survival. Table 1 summarizes the key results of the cited studies.

In addition, particle therapy (*e.g.*, proton or heavy ion therapy) can provide additional benefits compared to conventional EBRT, which uses X-rays in treating locally advanced HCC. Particle therapy

Table 1 Summary of key studies according to the treatment method

Ref.	Study type	Number of patients	Main outcomes
Radiotherapy studies			
Rim *et al*[33], 2018	Meta-analysis of observational studies	2111 with PVT	Pooled 1- and 2-yr OS: 43.8% and 22.3%, respectively (3DCRT) Pooled 1- and 2-year OS: 48.5% and 26.8%, respectively (SBRT) Grade 3 complications less than 5% to 10%
Huo *et al*[34], 2015	Comparative meta-analysis	2577 underwent TACE or RTx	TACE and RT had OS benefit compared with TACE alone ORs: 1.55, 1.91, 3.01, and 3.98 for 2-, 3-, 4-, and 5-yr OS rates, respectively
Yoon *et al*[35], 2018	Randomized trial	90 with major vascular invasion	TACE and RT had survival benefit compared with sorafenib Median OS 55 wk *vs* 43 wk, $P = 0.004$ Median PFS 31 wk *vs* 11.7 wk, $P < 0.001$
Lee *et al*[13], 2021	Observational study using national database	444 propensity-matched patients with PVT	Local treatment including RTx had survival benefit compared with no oncologic treatment Median OS: 8 mo *vs* 2 mo, $P < 0.001$ Median CSS: 8 mo *vs* 2 mo, $P < 0.001$ OS and CSS benefit persist in the CPC A and CPC B subgroups
Surgery studies			
Kokudo *et al*[41], 2016	Observational study using national database	2116 propensity-matched patients with PVT	Surgery had benefit compared with non-surgery Median OS: 2.45 yr *vs* 1.57 yr, $P < 0.001$ Surgery benefit was not observed in the Vp4 subgroup ($P = 0.242$)
Wang *et al*[42], 2016	Retrospective study	1580 with PVT underwent (1) surgery, (2) TACE, (3) TACE with sorafenib, or (4) TACE with RTx	Median OS: Cheng's type I: 15.9 *vs* 9.28 *vs* 12.0 *vs* 12.2 ($P < 0.001$) Cheng's type II: 12.5 *vs* 4.9 *vs* 8.9 *vs* 10.6 ($P < 0.001$) Cheng's type III: 6.0 *vs* 4.0 *vs* 7.0 *vs* 8.9 ($P = 0.001$)
Shi *et al*[43], 2010	Retrospective study	406 with PVT underwent surgery	Surgery showed better outcomes in Cheng's type I and type II (1-yr OS: 52% and 38%, respectively) PVT than type III and IV (1-yr OS: 25% and 18%, respectively)
Chen *et al*[44], 2006	Retrospective study	438 with PVT underwent surgery	Surgery yielded satisfactory results in Cheng's type I and II PVT (1- and 2-yr OS: 58.7% and 39.9%, respectively), not in types III and IV (1- and 2-yr OS: 39.5% and 20.4%, respectively)
Combined surgery and radiotherapy			
Chong *et al*[46], 2018	Retrospective study	26 underwent surgery following CCRT *vs* 18 underwent surgery alone	Surgery following CCRT had benefit on surgery alone Median DSS: 62 wk *vs* 15 wk, $P = 0.006$ Median DFS: 32 wk *vs* 3 wk, $P = 0.002$
Sun *et al*[47], 2019	Randomized trial	26 underwent surgery with adjuvant IMRT *vs* 26 surgery alone	Adjuvant IMRT significantly improved clinical outcomes Median OS: 18.9 mo *vs* 10.8 mo, $P = 0.005$ Median DFS: 9.1 mo *vs* 4.1 mo, $P = 0.001$
Wei *et al*[49], 2019	Randomized trial	82 neoadjuvant RT *vs* 82 surgery alone	1- and 2-yr OS: 75.2% and 27.4%, respectively (neoadjuvant RT) 1- and 2-yr OS: 43.2% and 9.4%, respectively (control) RT benefited Cheng's type I and II PVT as well as type III PVT
Li *et al*[50], 2016	Comparative study	45 neoadjuvant RT *vs* 50 surgery alone	Neoadjuvant RT decreased the rates of HCC recurrence [49% *vs* 88.7%, respectively ($P < 0.001$)] and increased overall survival [1-yr OS: 69% *vs* 35.6%, respectively ($P < 0.01$)]

PVT: Portal vein thrombosis; OS: Overall survival; 3DCRT: 3-dimensional conformal radiotherapy; SBRT: Stereotactic body radiotherapy; TACE: Transarterial chemoembolization; RTx: Radiotherapy; PFS: Progression-free survival; CSS: Cause-specific survival; CPC: Child-Pugh class; CCRT:

Concurrent chemoradiotherapy; DSS: Disease-specific survival; IMRT: Intensity-modulated radiotherapy.

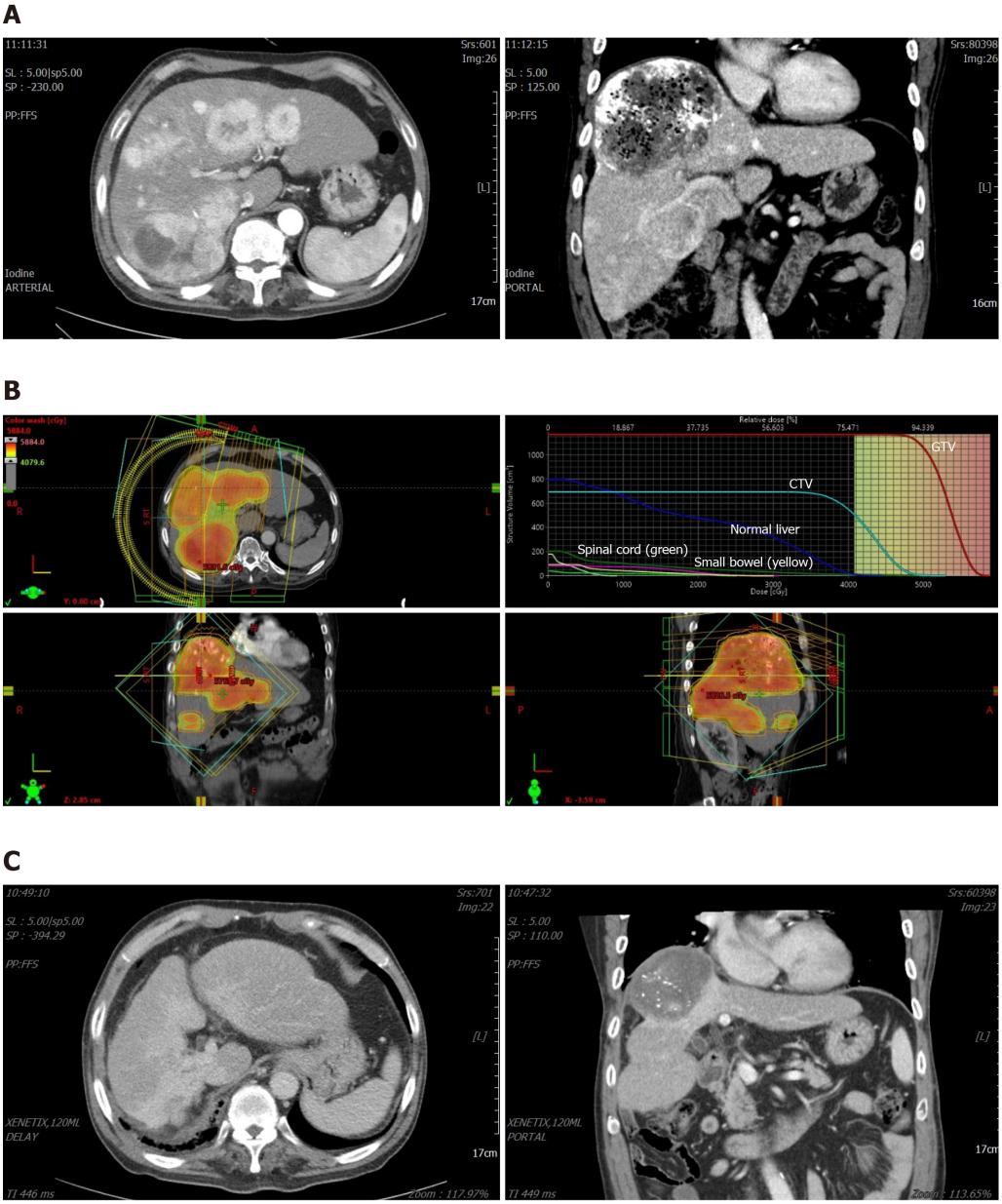


Figure 1 A case of a locally advanced hepatocellular carcinoma with portal thrombosis treated with radiotherapy. A: Multiple tumors noted in right lobe and segment 4, with a large tumor in segment 8, and involving right portal vein thrombosis; B: A dose-distribution of external radiotherapy plan. We prescribed 53 Gy/20F to gross tumor volume (red color wash in upper-left figure) with at least 42 Gy/20F were delivered to clinical target volume (green color wash in upper-left figure). Quantitative dose-histogram for specific organs is generated (upper-right figure). We planned to save at least 70% of normal liver to be irradiated less than 30 Gy; C: One year after radiotherapy and three times of transarterial chemoembolization, tumors were remised without active enhancing lesions. Liver function was maintained at Child-Pugh score A. GTV: Gross tumor volume; CTV: Clinical target volume.

is most similar to conventional EBRT in terms of the overall principle of causing cancer cell death. However, dose escalation and complication reduction could be achieved based on the physical characteristic called Bragg peak (*e.g.*, the phenomenon that energy deposits almost disappear after radiation passes through the body and progresses to a certain depth)[36]. Sanford *et al*[37] reported the benefit of proton therapy as compared to conventional EBRT for 133 unresectable HCC patients based on survival (median OS; 31 mo *vs* 14 mo, HR = 0.47, P = 0.008) and liver toxicities (odds ratio: 0.26, P = 0.03). Cheng *et al*[38] also reported the benefit of proton therapy as compared to conventional EBRT based on survival (HR 0.56, P = 0.032) and radiation-induced liver disease (11.8% *vs* 36.4%, P = 0.004), using a propensity-matched cohort. The current hurdle for using particle therapy is its accessibility; currently, there are about 110 particle therapy centers in operation worldwide, but most of them are in major developed countries such as the US, Japan, and Germany[39]. The financial burden of treatment due to

the high cost of equipment is also a problem to be resolved. However, the efficiency of EBRT could be greatly improved once these difficulties are gradually resolved.

In summary, EBRT can be used for the treatment of HCC by delivering a high dose of radiation and has technical advances. It is an effective palliative modality for HCC with portal invasion and is commonly performed along with TACE.

SURGICAL RESECTION OF HCC WITH PORTAL INVASION

Previously, surgery was not commonly performed in HCC patients with portal invasion because of the short life expectancy and therapeutic risks. Neither the EASL nor the AASLD guidelines suggest the performance of surgical resection as treatment for HCC with portal invasion[14,15]. However, East Asian countries, including China and Japan, have recently been actively performing surgery for portal invasion[40].

Kokudo *et al*[41] performed a key study to evaluate the efficacy of surgical resection in HCC patients with portal invasion using the nationwide data from Japan. Among 6474 HCC patients with PVT, approximately 2100 patients with Child-Pugh A liver function were matched using propensity scores (liver resection group vs. non-liver resection group). The liver resection group showed significantly longer survival (median: 2.45 years *vs* 1.57 years, $P < 0.001$). However, the OS benefit was not significant in the subgroup with Vp4 PVT ($P = 0.242$). Figure 2 shows an illustration of the two common classifications of PVT. Furthermore, R2 resection was performed in 60.5% of patients, and the 90-day mortality was 8.2% in the Vp4 PVT group. The authors recommended liver resection as the first-line of treatment for HCC with PVT in the first-order branch. Another large series conducted by four Chinese centers[42] which included 1572 HCC patients with PVT, reported similar results. The median survival times of the surgical group were 15.9 and 12.5 mo for PVT of type I and II in Cheng's classification, which were much better than those of their nonsurgical counterparts. However, for patients with Cheng's type III PVT, the TACE and RT group showed higher survival rate than the surgery group (8.9 mo *vs* 6.0 mo). Therefore, authors suggested that surgery should be considered for type I and II PVT, but TACE and RT should be recommended for type III PVT (PVT in the main trunk or contralateral branches). Other series from East Asian countries reported the feasibility of surgical resection in HCC patients with branch thrombosis, but this modality may lead to poor outcomes and increase the surgical risks in those with main PVT[43,44].

Based on the above studies, consensus guidelines in East Asia recommend the use of surgery for the treatment of HCC with portal invasion. The recent treatment guidelines of the Japan Society of Hepatology[45] suggested four possible options for HCC with major vessel invasion: three locoregional modalities including TACE, surgical resection, hepatic arterial infusion chemotherapy, and molecular-targeted therapy. They stated that it is difficult to provide a universal ranking for the four modalities; therefore, the four modalities are recommended in parallel. On the contrary, the Chinese Expert guidelines for HCC with PVT recommend surgical resection as a preferred option for patients with Child-Pugh A, PVT type I and II based on Cheng's classification (branch PVT), and good performance. Type III PVT cases are recommended to undergo surgery after downstaging *via* EBRT or TACE[23].

In summary, surgical treatment is being actively performed for HCC with PVT, especially in East Asia. Although surgical resection is a considerable modality for HCC with branch PVT (types I and II based on Cheng's classification; Vp1-3 based on the Japanese classification), this modality can lead to poor outcomes and increase the risk for perioperative complications when used in patients with main PVT. Table 1 summarizes the key studies related to this topic.

PROMISING RESULTS OF SURGERY AND RADIOTHERAPY AS COMBINATION TREATMENT

In cancer treatment, the application of radiotherapy before and after surgical treatment to lower the recurrence and survival rates is a widely used method. Previously, patients with HCC with portal invasion were deemed to have a dismal prognosis; therefore, active treatment combining surgery and EBRT has not been widely accepted. However, several researchers have recently reported promising outcomes of neo- or adjuvant EBRT.

The multidisciplinary team of Yonsei University[46] reported their experience of trimodality treatment for HCC with portal invasion, which is neoadjuvant CCRT (HAIC plus EBRT) yielding downstaging and surgical resection. Patients who underwent trimodality treatment had a median disease-specific survival of 62 mo, while those who underwent resection alone had a median disease-specific survival of only 15 mo ($P = 0.006$). Sun *et al*[47] from the Eastern Hepatobiliary Surgery Hospital performed a randomized study to investigate the benefit of adjuvant IMRT in HCC patients with PVT (adjuvant RT *vs* surgery alone). The adjuvant radiotherapy group showed significantly higher disease-free survival (DFS) (median: 9.1 mo *vs* 4.1 mo, $P = 0.001$) and OS (median: 18.9 vs. 10.8 mo, $P = 0.001$).

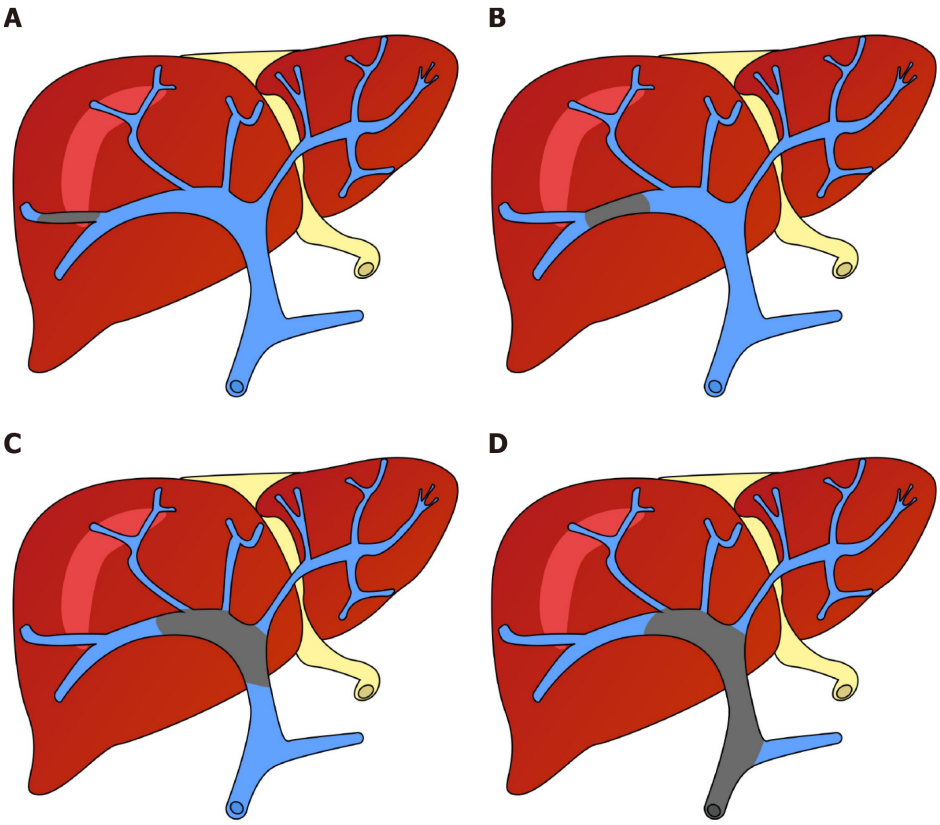


Figure 2 Illustration of two systems categorizing portal vein thrombus. A: Invasion of the second-order branch of the portal vein: VP2 in the liver cancer study group of Japan (*e.g.*, Vp1 denotes the invasion distal to the second-order branch) and Cheng's classification type I; B: Invasion of the first-order branch: VP3 and Cheng's type II; C: Invasion of the main branch and/or bilateral first-order branches: VP4 and Cheng's type III; D: Invasion of the superior mesenteric vein: Cheng's type IV.

The most common complications after RT were fatigue or anorexia, and grade 3 gastrointestinal complications occurred in 2 of 26 patients (7.7%). Grade 4 or higher adverse effects have not been reported. Although OS difference was significant in subgroup analyses among patients with Cheng's type I or II PVT (median 20.7 mo *vs* 11.7 mo, $P = 0.008$), due to the limited number of patients (only 6 and 7 patients had Cheng's type III or IV PVT, respectively, in both arms), the difference was not considered significant in the subgroup with PVT at main branch or trunk. Soin *et al* [48] reported encouraging results that comparable survival was achieved in HCC patients with PVT after down-staging, including SBRT, and liver transplantation, to those without PVT who underwent transplantation (5-year OS 57% *vs* 65%, $P = 0.06$).

Wei *et al*[49] performed a cornerstone study related to this topic, randomizing 164 patients into the neoadjuvant EBRT and surgery group and surgery groups. The 1- and 2-year OS rates in the neoadjuvant arm were 75.2% and 27.4%, whereas those in the surgery arm were 43.1% and 9.4%, respectively ($P < 0.001$). EBRT improved the OS and DFS of patients with type II PVT ($P = 0.01$ and $P = 0.016$, respectively) and those with type III PVT ($P < 0.001$ and $P = 0.002$, respectively), according to Cheng's classification. No significant difference was observed in the perioperative complications between the two groups, although a few more grade 3 or 4 complications were reported after RT (two cases of abdominal hemorrhage in the neoadjuvant EBRT arm and none in the surgery arm). In a previous non-randomized comparative study on HCC with main PVT (type III based on Cheng's classification), Li *et al*[50] investigated the benefit of neoadjuvant radiotherapy. The 1- and 2-year OS rates in the neoadjuvant group were 69% and 20.4%, whereas those in the surgery group were 35.6% and 0%, respectively ($P < 0.01$). The recurrence rates were 49% and 88.7% at 6 mo in the neoadjuvant and surgery groups, respectively ($P < 0.001$).

In summary, recent studies demonstrated that combining neo- or adjuvant EBRT and surgery could further improve the oncologic outcomes of HCC patients with portal invasion, possibly those with main PVT. Table 1 presents a list of related studies.

FUTURE PERSPECTIVE AND SUMMARY

Until the 2000s, there were limited practical treatment methods for HCC with portal invasion. Since the

mid-2000s, sorafenib, the first effective systemic agent for unresectable HCC, has been established. In recent decades, aside from TACE, which is the most commonly used locoregional modality, other methods including EBRT, TARE, HAIC, and surgery have also been attempted. Furthermore, the use of a novel systemic modality (atezolizumab-bevacizumab)[51] that surpassed sorafenib in terms of survival and tumor responses, which had been established as a standard systemic agent for 15 years, was reported in 2020. Atezolizumab-bevacizumab is a combination of anti PD-L1 (an immune checkpoint inhibitor) and anti-vascular endothelial growth factor (VEGF) (a tumor microenvironment-modulating agent). As the anti-VEGF therapy reverses the VEGF-mediated immune suppression and increases the T-cell infiltration in tumors, the efficacy of anti PD-L1 could be enhanced[51]. Radiation therapy also enhances the performance of tumor antigen presentation and T-cell infiltration in the tumors[52]. In addition, radiation itself induces the sensitization of tumor cells to immune-mediated cell death by upregulating FAS expression. Therefore, the combination of immune checkpoint inhibitors and radiation therapy could be a promising treatment for HCC due to its synergistic effect. In addition, advances in understanding tumor immunity have resulted in new emerging immunotherapies. For example, CD4+ CD25+ regulatory T cells have a well-established immunosuppressive role in the HCC microenvironment and express various chemokine receptors and surface molecules such as PD-1, CTLA4 and others[53]. They can potentially be direct or indirect targets for newly emerging immune checkpoint inhibitor immunotherapy. Future clinical studies investigating the efficacy and feasibility of novel immunotherapy in combination with EBRT are necessary.

CONCLUSION

Although HCC with portal invasion is considered to have a limited benefit from surgery, pioneering researchers have obtained promising outcomes, and recent studies have demonstrated that the addition of EBRT can further increase the treatment efficiency. If effective novel systemic agents, surgery, and EBRT are used in an appropriate combination, the prognosis of HCC with portal invasion can be significantly improved. In other words, we believe that the most potent anticancer modalities known to date, the tripartite collaboration of chemotherapy, surgery, and radiotherapy, commonly used in the treatment of other solid cancers, will be used as a new standard treatment for HCC with portal invasion in the near future. However, clinical trials are warranted to evaluate the efficacy of such collaborations.

FOOTNOTES

Author contributions: Rim CH contributed to conceptualization; Lee HY supervised the study; Choe JW and Rim CH wrote the original draft; Choe JW, Lee HY and Rim CH reviewed and edited the manuscript; All authors have read and agreed to the published version of the manuscript.

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Country/Territory of origin: South Korea

ORCID number: Jung Wan Choe 0000-0003-0634-5141; Hye Yoon Lee 0000-0001-9077-1412; Chai Hong Rim 0000-0001-7431-4588.

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ORIGINAL ARTICLE

Retrospective Study

Clinical online nomogram for predicting prognosis in recurrent hepatolithiasis after biliary surgery: A multicenter, retrospective study

Tian Pu, Jiang-Ming Chen, Zi-Han Li, Dong Jiang, Qi Guo, Ang-Qing Li, Ming Cai, Zi-Xiang Chen, Kun Xie, Yi-Jun Zhao, Cheng Wang, Hui Hou, Zheng Lu, Xiao-Ping Geng, Fu-Bao Liu

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Tian Pu, Jiang-Ming Chen, Zi-Han Li, Dong Jiang, Qi Guo, Ang-Qing Li, Zi-Xiang Chen, Kun Xie, Yi-Jun Zhao, Xiao-Ping Geng, Fu-Bao Liu, Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Ming Cai, Cheng Wang, Department of General Surgery, The First Affiliated Hospital of the University of Science and Technology of China, Hefei 230001, Anhui Province, China

Hui Hou, Department of General Surgery, The Second Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Zheng Lu, Department of General Surgery, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233030, Anhui Province, China

Corresponding author: Fu-Bao Liu, MD, PhD, Chief Doctor, Professor, Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, No. 218, Jixi Road, Hefei 230022, Anhui Province, China. lancetlfb@126.com

Abstract

BACKGROUND

Methods for predicting the prognosis of patients undergoing surgery for recurrent hepatolithiasis after biliary surgery are currently lacking.

AIM

To establish a nomogram to predict the prognosis of patients with recurrent hepatolithiasis after biliary surgery.

METHODS

In this multicenter, retrospective study, data of consecutive patients in four large medical centers who underwent surgery for recurrent hepatolithiasis after biliary surgery were retrospectively analyzed. We constructed a nomogram to predict the prognosis of recurrent hepatolithiasis in a training cohort of 299 patients, following which we independently tested the nomogram in an external validation cohort of 142 patients. Finally, we used the concordance index (C-index), calibration, area under curve, decision curve analysis, clinical impact curves, and visual fit indices to evaluate the accuracy of the nomogram.

RESULTS

Multiple previous surgeries [2 surgeries: Odds ratio (95% confidence interval), 1.451 (0.719-2.932); 3 surgeries: 4.573 (2.015-10.378); ≥ 4 surgeries: 5.741 (1.347-24.470)], bilateral hepatolithiasis [1.965 (1.039-3.717)], absence of immediate clearance [2.398 (1.304-4.409)], neutrophil-to-lymphocyte ratio ≥ 2.462 [1.915 (1.099-3.337)], and albumin-to-globulin ratio ≤ 1.5 [1.949 (1.056-3.595)] were found to be independent factors influencing the prognosis. The nomogram constructed on the basis of these variables showed good reliability in the training (C-index: 0.748) and validation (C-index: 0.743) cohorts. Compared with predictions using traditional classification models, those using our nomogram showed better agreement with actual observations in the calibration curve for the probability of endpoints and the receiver operating characteristic curve. Dichloroacetate and clinical impact curves showed a larger net benefit of the nomogram.

CONCLUSION

The nomogram developed in this study demonstrated superior performance and discriminative power compared to the three traditional classifications. It is easy to use, highly accurate, and shows excellent calibration.

Key Words: Gallstones; Reoperation; Risk factors; Nomogram; Prognosis; Model

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Core Tip: This is a multicenter, retrospective study to establish a nomogram to predict the prognosis of patients with recurrent hepatolithiasis after biliary surgery. After this, an online calculator was developed, and the nomograms are freely available on the internet. We used the concordance index, calibration, area under curve, decision curve analysis, clinical impact curves, and visual fit indices to evaluate the accuracy of the nomogram. Compared with predictions using traditional classification models, those using our nomogram showed better agreement with actual observations.

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INTRODUCTION

Hepatolithiasis is mostly prevalent in East and Southeast Asia, and the incidence in China is the highest in the world[1]. Although treatment strategies for hepatolithiasis have been improving, the overall treatment rate and prognosis remain poor because of the long course, complex pathological changes, high incidence of postoperative complications, and high recurrence rate. Sporadic cases of hepatolithiasis have been reported in Western countries, and with the increasing immigration from Asian countries, hepatolithiasis has become increasingly prevalent in the West[2-4]. The prevalence of this disease is 30%-50% in East Asia and 0.6%-1.3% in Western countries[5].

The treatment of hepatolithiasis involves pharmacologic, endoscopic, and surgical approaches. Surgery is the most effective treatment. However, the course of the disease varies, and stones can easily remain in the surgery and recur at a later date. Patients with recurrent hepatolithiasis who undergo multiple biliary surgeries often experience varying degrees of abdominal adhesions, causing greater difficulties and increasing the risks for surgery. Thus, surgeons should focus on improving the prognosis and quality of life of patients.

Recurrent hepatolithiasis is defined as hepatolithiasis with a history of biliary tract surgery for different reasons. Some classification systems for hepatolithiasis have been established, such as classification based on clinical manifestations[3], Nakayama[6]'s classification based on the distribution of stones, Tsunoda *et al*[7]'s classification based on dilatation or stenosis, and the Chinese Classification model proposed by the Biliary Study Group of the Chinese Medical Association[8]. Despite their wide acceptance, these classification models are too complicated to implement in guiding clinical treatment. Thus, clinical classification of hepatolithiasis has practical significance in guiding treatment decisions and predicting patient prognoses. Nomograms are statistical tools that enable simultaneous consideration of various factors to facilitate visualization of prognoses. Moreover, nomograms offer many advantages, including personalized evaluation, user-friendliness, and ease of comprehension[9].

Considering the absence of a prediction model for the quality of life of patients with recurrent hepatolithiasis, this study aimed to establish a nomogram for predicting the prognosis of patients with recurrent hepatolithiasis after biliary surgery.

MATERIALS AND METHODS

Clinical and prognostic data of patients with recurrent hepatolithiasis who underwent surgery between January 2015 and December 2020 at the Departments of Hepatopancreatobiliary Surgery in four medical centers were retrospectively analyzed for evaluating patients' quality of life. The patients from the First Affiliated Hospital of Anhui Medical University constituted the training cohort, while those from the Second Affiliated Hospital of Anhui Medical University, the First Affiliated Hospital of University of Science and Technology of China and the First Affiliated Hospital of Bengbu Medical College served as the validation cohort. All hospitals are high-volume surgical centers employing similar therapeutic approaches for hepatolithiasis.

The diagnosis of hepatolithiasis was confirmed by preoperative imaging examinations and intraoperative findings. Recurrent hepatolithiasis was diagnosed when the patient had a history of biliary surgery. These patients had already undergone at least one bile duct surgery for hepatolithiasis. Patients from the four centers were included if they met the following inclusion criteria: (1) A history of at least one biliary surgery; (2) Confirmation of stones by preoperative imaging examination; (3) Confirmation of intrahepatic cholangiolithiasis during the procedure; and (4) A preoperative liver function of Child-Pugh grade A or initial grade B that improved to grade A. The exclusion criteria were as follows: (1) Hepatolithiasis occurring within 6 mo after the last biliary tract surgery; (2) No history of surgical treatment; (3) History of abdominal surgery not involving the biliary tract system; (4) Malignant tumor on postoperative pathological evaluation; and (5) Patchy clinical or follow-up data. All clinical data were screened and collected in a computerized database by a specialized research assistant. This retrospective study was conducted in accordance with the declaration of Helsinki and was approved by the institutional ethics committees (Quick-PJ2021-08-19). All included patients or their relatives provided written informed consent before their data were analyzed.

Preoperative evaluation

Under the same preoperative evaluation protocol across all centers, the patients underwent blood tests, including routine blood counts and analysis of blood biochemistry, hemostatic function, immunological markers, and tumor markers. All patients underwent at least two imaging examinations, including ultrasound (US), computed tomography (CT), magnetic resonance imaging, or magnetic resonance cholangiopancreatography (MRCP), which provided information on the location of stones, biliary strictures, or liver atrophy. Definitive planning of the procedure was performed according to the findings of imaging studies. For patients with complex bilateral hepatolithiasis or those expected to undergo extensive liver resection, the ratio of the future remnant liver to total functional liver volume was calculated by volumetric CT scans or three-dimensional visualization techniques, and the indocyanine green 15-min retention rate was measured to evaluate the safety of surgeries.

Reoperative procedures

Patients who failed to reach Child-Pugh grade A before surgery underwent liver protection and supportive treatment. All surgeries were performed by experienced hepatobiliary surgeons. Patients with a history of repeated surgeries often have abdominal adhesions. After relieving the abdominal adhesions, a detailed surgical plan was created on the basis of the intraoperatively confirmed stone location, bile duct stenosis, liver atrophy, and function of the sphincter of Oddi (SO). The main surgical objective was to remove as many stones as possible and choose the appropriate method for biliary drainage. Routine intraoperative flexible choledo-choscopy was performed after longitudinal incision of the common bile duct and removal of visible stones to determine the stone distribution and identify residual stones, which were directly extracted with a stone basket when needed. Then, SO function was evaluated, and the biliary drainage method was chosen based on SO function and the presence of residual stones; external T tube drainage was chosen for normal SO without residual stones, and cholangioenterostomy was chosen for SO laxity without residual stones. If residual stones could not be prevented, cholangioenterostomy and T-tube drainage were performed simultaneously[10]. Hepatectomy should be performed when bile duct stones are located within one liver lobe accompanied by atrophy or fibrosis, multiple liver abscesses secondary to bile duct infection, and suspected malignant masses. We applied the Pringle maneuver to occlude the blood inflow to the liver if necessary. Choledochoscopy was performed again after hepatic lobe resection to check for residual stones and to assess whether stones were cleared immediately. Bile acid was collected during the surgery and sent for bacterial culture and drug sensitivity testing in all patients.

Postoperative management

Standardized and meticulous postoperative patient management was performed in all patients at an early stage, including monitoring of vital signs, proper tissue perfusion, and nutritional support. Gastric acid secretion inhibitors and broad-spectrum antibiotics were administered immediately after the surgery, and antibiotics were adjusted according to the results of bile acid culture. Liver function tests were performed at 1, 3, and 7 d after the surgery. According to the Clavien-Dindo classification system, complications occurring within 90 d postoperatively were classified as grades I-V. Before discharge, all patients underwent abdominal CT examination again to further confirm whether the stone was removed immediately during the surgery. Patients who undergo external drainage of the T tube should undergo cholangiography or choledochoscopy after discharge to confirm or remove residual stones. In the present study, for patients with immediate stone residue, we usually performed choledochoscopy through the sinus of the T tube at 6-8 wk after the surgery; this was performed several times until the stone was removed or could not be removed by any means. For patients with immediate clearance, we performed T-tube cholangiography at 2 wk after surgery. In case a residual stone was observed, we performed choledo-choscopy as described above.

Follow-up

All patients underwent regular postoperative follow-up by the same team of surgeons in the hepato-biliary outpatient clinics or through telephone interviews at 2-3 mo after discharge. Follow-up evaluations included assessment of clinical symptoms and signs, routine blood tests, liver-function assessments, and US, CT, or MRCP to observe residual or recurrent stones. Postoperative residual stones were defined as stones that could not be removed by any method and were confirmed by US, CT, or MRCP 3 mo postoperatively[11]. Prognosis was evaluated according to the Terblanche criteria[12]. The patients were evaluated from 30 d to the end of the follow-up: (1) Grade I, no bile duct-related symptoms; (2) Grade II, occasional bile duct-related symptoms requiring no treatment; (3) Grade III, obvious bile duct-related symptoms requiring treatment; or (4) Grade IV, presence of anastomotic stricture or formation of bile duct stones requiring surgical intervention and causing disease-related cancer or death. Ter-blanche grades III and IV were considered to indicate a poor prognosis, which was the study endpoint.

Statistical analysis

Continuous variables are expressed as mean ± SD for normally distributed variables or median (interquartile range) for non-normally distributed variables, and appropriate statistical tests (the independent samples *t*-test or the Mann-Whitney *U* test) were used. Categorical variables are expressed as number (*n*) or proportion (%) and compared using the χ^2 test or Fisher's exact test, as appropriate. Assigned cutoff values for continuous variables were derived from the Youden index[13]. Univariate logistic regression was used to determine the independent risk factors related to the prognosis of patients with recurrent hepatolithiasis after multiple biliary surgeries in the training cohort. Multivariate logistic regression was conducted using variables with clinical meaning or statistical significance in the univariate analyses. A nomogram for the prognosis of patients with recurrent hepato-lithiasis after biliary surgery was created based on a multivariate logistic regression model. The performance of the nomogram was evaluated using the concordance index (C-index) and calibration plots with bootstrap samples. The C-index is a numerical measure of discriminative ability, and calibration plots are graphical evaluations of predictive ability that compare observed probabilities with nomogram-predicted probabilities. The area under the curve (AUC) of the receiver operating charac-teristic (ROC) curves and quality indices of models[14] in the training cohort and the external validation cohort were used to assess the predictive accuracy of the model in comparison with the three traditional classifications. The clinical usefulness of the nomogram was examined by determining the net benefit by using decision curve analysis (DCA)[15]. Clinical impact curves were also analyzed to demonstrate the predictive accuracy and clinical usefulness of the nomogram. The accuracy of the optimal cutoff value was assessed based on sensitivity, specificity, and predictive values. Statistical analyses were performed using R version 4.0.5, and SPSS version 23.0. Tests were 2-sided, and statistical significance was set at $P < 0.05$.

RESULTS

Baseline clinical characteristics and prognosis

Data of 943 consecutive patients who underwent surgical treatment for hepatolithiasis at the First Affiliated Hospital of Anhui Medical University between January 2015 and December 2020 were collected continuously. Among them, 363 patients (38.5%) with a history of biliary tract surgeries were classified as having recurrent hepatolithiasis. Of these 363 patients, 64 (17.6%) who did not fulfill the inclusion criteria were excluded: 28 were admitted to the hospital with a malignant tumor, 9 had a history of other abdominal surgery, 1 died in the perioperative period, 22 had incomplete clinical or

follow-up data, and 4 died of other causes after surgery. Ultimately, 299 (82.4%) patients were identified as the training cohort. Using the same criteria, 142 patients from the Second Affiliated Hospital of Anhui Medical University (57 cases), the First Affiliated Hospital of the University of Science and Technology of China (51 cases), and the First Affiliated Hospital of Bengbu Medical College (34 cases) were included in the external validation cohort. The two cohorts showed significant differences in the pre-, intra-, and postoperative clinical characteristics, as shown in Tables 1 and 2.

All patients had a history of multiple (1-6) biliary tract surgeries 1-55 years before this surgery. The training cohort included 199 (66.6%), 57 (19.1%), 33 (11.0%), and 10 (3.3%) patients who underwent 1, 2, 3, and ≥ 4 surgeries, respectively. In total, 167 (55.9%) patients had a history of cholecystectomy only, without hepatectomy or biliary drainage, while 61 (20.4%) and 42 (14.0%) patients had a history of hepatectomy and cholangioenterostomy, respectively. The validation cohort included 101 (71.1%), 29 (20.4%), 9 (6.3%), and 3 (2.1%) patients who underwent 1, 2, 3, and ≥ 4 surgeries, respectively; 167 (55.9%) patients had previously undergone only cholecystectomy, while 23 (16.2%) and 11 (7.7%) patients had a history of hepatectomy and cholangioenterostomy, respectively. Gall bladder removal was performed in the first (93.0%) or second (7.0%) surgeries for all patients. The appropriate surgical method was selected on the basis of preoperative evaluation and intraoperative conditions, and anastomosis reconstruction was performed depending on the presence of stenosis. In the training cohort, 229 (76.6%) and 283 (94.6%) patients achieved immediate and final clearance, respectively, and 129 (43.1%), 78 (26.1%), 66 (22.1%), and 26 (8.7%) patients showed Terblanche grades I-IV, respectively. In the validation cohort, 115 (81.0%) and 137 (96.5%) patients showed immediate and final clearance, respectively, and 53 (37.3%), 43 (30.3%), 34 (23.9%), and 12 (8.5%) patients showed Terblanche grades I-IV, respectively.

Uni- and multivariate analysis in the training cohort

The results of univariate and multivariate analyses of prognosis based on common variables, including demographic data, clinical symptoms, surgical histories, serologic data, and operative data, in the training cohort are listed in Table 3. The optimal cutoff values of the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio were determined as described above. Factors that significantly affected prognosis in the univariate analysis were subjected to multivariate analysis, which demonstrated that more previous surgeries, bilateral hepatolithiasis, lack of immediate clearance, NLR ≥ 2.462, and albumin-to-globulin ratio (AGR) ≤ 1.5 were independent risk factors for a poor prognosis in patients with recurrent hepatolithiasis.

Development and evaluation of the predictive model

The independent risk factors associated with prognosis were then used to construct a nomogram for estimating the risk of poor prognosis (Figure 1). The nomogram demonstrated good predictive performance in estimating the risk of poor prognosis after reoperation for recurrent hepatolithiasis [C-index, 0.748; 95% confidence interval (CI): 0.687-0.810] in the training cohort and 0.743 (95%CI: 0.654-0.832 in the validation cohort). The constructed model was internally validated using the bootstrap validation method (n = 1000) to reduce the overfitting bias, and the calibration plots in the internal and external validations demonstrated good consistency between the observed and predicted probabilities. The predicted curves approximately overlapped with the reference curves, indicating good performance of the nomograms in both cohorts (Figure 2). The Brier score for overall performance, which assesses the difference between the observed and predicted values, was 0.175 (values closer to 0 indicated better predictive ability). The calibration slope, which assesses the agreement between the observed and predicted values, was 1.0 (values closer to 1 indicate better performance)[16]. An online calculator was developed, and the nomograms are freely available at https://ahmuptt.shinyapps.io/DynNomapp/ for prognosis.

Comparison of the performance of the nomogram and traditional classifications models

The discriminative performance of the three traditional classification models (Hepatolithiasis Research Group, Tsunoda classification, and Chinese Medical Association) were compared with that of the nomogram established in this study through ROC analyses (Figure 3). The AUC of the nomogram and the three classification models were 0.750, 0.544, 0.552, and 0.565 in the training cohort and 0.754, 0.608, 0.508, and 0.586 in the validation cohort, respectively. Thus, the nomogram showed better accuracy in predicting the prognosis for recurrent hepatolithiasis after reoperation. The optimal cutoff value of the nomogram total score was 77.5 in the ROC curve considering the maximum Youden index value, and the sensitivity and specificity for differentiating between good and poor prognoses were 62.0% and 79.2%, respectively. Using this cutoff value, patients with total nomogram scores of < 77.5 points or ≥ 77.5 points were classified as having a low or high risk of poor prognosis. In Figure 4, DCA graphically shows that the use of the nomogram to predict prognosis when the threshold probability ranged from 0.2 to 0.6 added more net benefit than the other three traditional classifications. The clinical impact curves of the nomogram indicated that the models had remarkable predictive power. Finally, in comparison with the other three traditional classifications, the fit indices of the nomogram are also visually reported in Figure 5.

Table 1 Preoperative clinical characteristics of patients with recurrent hepatolithiasis after biliary surgery

Characteristic	Training cohort	Validation cohort	*P* value
	N = 299	*N* = 142	
Sex			0.785
Male	93 (31.1%)	46 (32.4%)	
Female	206 (68.9%)	96 (67.6%)	
Age (yr)			0.613
< 60	153 (51.2%)	69 (48.6%)	
≥ 60	146 (48.8%)	73 (51.4%)	
BMI	21.72 ± 2.87	22.19 ± 2.96	0.317
Abdominal pain			0.204
No	39 (13.0%)	25 (17.6%)	
Yes	260 (87.0%)	117 (82.4%)	
Fever			0.162
No	175 (58.5%)	93 (65.5%)	
Yes	124 (41.5%)	49 (34.5%)	
Jaundice			0.060
No	227 (75.9%)	119 (83.8%)	
Yes	72 (24.1%)	23 (16.2%)	
Number of previous surgeries			0.374
1	199 (66.6%)	101 (71.1%)	
2	57 (19.1%)	29 (20.4%)	
3	33 (11.0%)	9 (6.3%)	
≥ 4	10 (3.3%)	3 (2.1%)	
Previous hepatectomy			0.293
No	238 (79.6%)	119 (83.8%)	
Yes	61 (20.4%)	23 (16.2%)	
Previous cholangioenterostomy			0.057
No	257 (86.0%)	131 (92.3%)	
Yes	42 (14.0%)	11 (7.7%)	
NLR			0.453
< 2.462	188 (62.9%)	84 (59.2%)	
≥ 2.462	111 (37.1%)	58 (40.8%)	
< PLR			0.804
173.74	237 (79.3%)	114 (80.3%)	
≥ 173.74	62 (20.7%)	28 (19.7%)	
AGR			0.430
> 1.5	104 (34.8%)	44 (31.0%)	
≤ 1.5	195 (65.2%)	98 (69.0%)	
TB (μmol/L)			0.262
< 34.2	249 (83.3%)	112 (78.9%)	
≥ 34.2	50 (16.7%)	30 (21.1%)	
ALT (IU/L)			0.474

< 50	181 (60.5%)	91 (64.1%)	
≥ 50	118 (39.5%)	51 (35.9%)	
AST (IU/L)			0.522
< 40	180 (60.2%)	90 (63.4%)	
≥ 40	119 (39.8%)	52 (36.6%)	
ALP (IU/L)			0.053
< 200	180 (60.2%)	99 (69.7%)	
≥ 200	119 (39.8%)	43 (30.3%)	
GGT (IU/L)			0.182
< 150	146 (48.8%)	79 (55.6%)	
≥ 150	153 (51.2%)	63 (44.4%)	
HBsAg			0.107
Negative	271 (90.6%)	135 (95.1%)	
Positive	28 (9.4%)	7 (4.9%)	
CA19-9 (U/mL)			0.428
< 34	208 (69.6%)	104 (73.2%)	
≥ 34	91 (30.4%)	38 (26.8%)	
Hepatolithiasis research group			0.089
I	19 (6.4%)	18 (12.7%)	
II	152 (50.8%)	67 (47.2%)	
III	100 (33.4%)	49 (34.5%)	
IV	28 (9.4%)	8 (5.6%)	
Tsunoda classification			0.052
I	17 (5.7%)	19 (13.4%)	
II	166 (55.5%)	74 (52.1%)	
III	100 (33.4%)	43 (30.3%)	
IV	16 (5.4%)	6 (4.2%)	
Chinese medical association			0.295
I	238 (79.6%)	123 (86.6%)	
IIa	30 (10.0%)	8 (5.6%)	
IIb	25 (8.4%)	8 (5.6%)	
IIc	6 (2.0%)	3 (2.1%)	

BMI: Body mass index; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; AGR: Albumin-to-globulin ratio; TB: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; HbsAg: Hepatitis B surface antigen; CA19-9: Carbohydrate antigen 19-9.

DISCUSSION

Our study indicated that for patients with recurrent hepatolithiasis following multiple biliary tract surgeries, multiple previous surgeries, bilateral hepatolithiasis, failure to clear the stones immediately, preoperative NLR ≥ 2.462, and preoperative AGR ≤ 1.5 were significant predictors. These factors combined the patients' medical history, preoperative imaging and serological data, and intraoperative outcomes to comprehensively quantify the prognosis of patients in a concise and intuitive manner. Multiple validation methods also indicated that the model had sufficient statistical power to predict the prognosis. Moreover, considering the inconvenience of traditional nomograms for clinical use, an online version of the nomogram was built, which could be easily accessed using computers, smartphones, or other mobile devices, thereby greatly improving clinical practicability.

Table 2 Intra- and postoperative clinical characteristics of patients with recurrent hepatolithiasis after biliary surgery

Characteristic	Training cohort	Validation cohort	*P* value
	N = 299	*N* = 142	
Operation duration (h)			0.168
≤ 2	66 (22.1%)	38 (26.8%)	
2-4	122 (40.8%)	64 (45.1%)	
> 4	111 (37.1%)	40 (28.2%)	
Liver cirrhosis			0.690
No	261 (87.3%)	122 (85.9%)	
Yes	38 (12.7%)	20 (14.1%)	
Hepatic atrophy			0.068
No	124 (41.5%)	72 (50.7%)	
Yes	175 (58.5%)	70 (49.3%)	
Intrahepatic stenosis			0.182
No	238 (79.6%)	105 (73.9%)	
Yes	61 (20.4%)	37 (26.1%)	
Extrahepatic stones			0.813
No	64 (21.4%)	29 (20.4%)	
Yes	235 (78.6%)	113 (79.6%)	
Hepatectomy			0.084
No	101 (33.8%)	60 (42.3%)	
Yes	198 (66.2%)	82 (57.7%)	
Bilateral hepatolithiasis			0.074
No	238 (79.6%)	123 (86.6%)	
Yes	61 (20.4%)	19 (13.4%)	
Drainage mode			0.125
External T tube drainage	205 (68.6%)	109 (76.8%)	
Cholangioenterostomy	44 (14.7%)	19 (13.4%)	
Combined drainage	50 (16.7%)	14 (9.9%)	
Function of the SO			0.521
Normal	130 (43.5%)	72 (50.7%)	
Dysfunction	62 (20.7%)	27 (19.0%)	
Nonfunctional	83 (27.8%)	32 (22.5%)	
Resected	24 (8.0%)	11 (7.7%)	
Intraoperative bleeding (mL)			0.465
< 400	285 (95.3%)	133 (93.7%)	
≥ 400	14 (4.7%)	9 (6.3%)	
Blood transfusion			0.112
No	258 (86.3%)	130 (91.5%)	
Yes	41 (13.7%)	12 (8.5%)	
TB after operation (μmol/L)			0.131
< 34.2	232 (77.6%)	119 (83.8%)	
≥ 34.2	67 (22.4%)	23 (16.2%)	

Bile culture			0.471
Negative	196 (65.6%)	98 (69.0%)	
Positive	103 (34.4%)	44 (31.0%)	
Clavien-Dindo classification			0.784
< III	286 (95.7%)	135 (95.1%)	
≥ III	13 (4.3%)	7 (4.9%)	
Hospitalization expenses	48272 ± 22537	44933 ± 25354	0.164
Immediate clearance			0.298
Yes	229 (76.6%)	115 (81.0%)	
No	70 (23.4%)	27 (19.0%)	
Final clearance			0.399
Yes	283 (94.6%)	137 (96.5%)	
No	16 (5.4%)	5 (3.5%)	

SO: Sphincter of Oddi; TB: Total bilirubin.

Reoperation remains the preferred treatment for patients with recurrent hepatolithiasis. Satisfactory stone-clearance rates can be achieved through comprehensive preoperative evaluation, meticulous intraoperative exploration, and postoperative T-tube angiography with or without choledochoscopy. All patients in this study showed recurrent hepatolithiasis after biliary tract surgery. The immediate and final clearance rates were 78.0% and 95.2%, respectively, which were lower than those reported in previous studies that did not distinguish between primary and recurrent hepatolithiasis[17-19].

In addition to requiring traumatic wounds, repeated surgeries impose an enormous psychological and economic burden on patients and their families. Moreover, consi-dering the difficulty in guaran-teeing the prognosis, patients will inevitably blame the surgeon, raising the possibility of doctor-patient conflict. Thus, accurate evaluation of the condition of patients with recurrent hepatolithiasis and provision of references for clinical efficacy are essential. However, the existing classification models for hepatolithiasis cannot describe the curative effect of prospective evaluation. In this study, we collected clinical and follow-up data of patients who underwent reoperation for recurrent hepatolithiasis at four large hepatobiliary centers and established and validated a nomogram model based on multicenter data. To the best of our know-ledge, this is the first study to establish a predictive model for prognosis follow-ing multiple biliary tract surgeries in patients with recurrent hepatolithiasis after initial biliary surgery.

The nomogram clearly showed that the risk of a worse prognosis increased with the number of previous surgeries. The serious abdominal adhesions caused by repeated surgeries and the resultant disconnection and anastomosis of the tube will lead to complicated intraoperative conditions, making it difficult to excise the lesion and clear the stones accurately and increasing the possibility of a poor prognosis. Moreover, the nomogram showed that the model score for cases with three previous surgeries (67 points) was much higher than that for cases with two previous surgeries (33 points), suggesting that three previous surgeries significantly increased the possibility of a poor prognosis. Thus, in patients with an extended surgical history for the treatment of hepatolithiasis, a curative procedure and good quality of life are difficult to achieve, and such patients may experience prolonged disease in addition to the tremendous economic pressure caused by the repeated surgeries. Therefore, the benefits and disadvantages of repeat surgeries for patients should be weighed with care. We propose that conservative or non-open surgical treatment should be considered as the first choice of treatment, with conventional open surgery considered the second choice, for patients with a total nomogram score of > 77.5 or patients who meet the following criteria: (1) ≥ 3 previous surgeries; (2) No obvious bile duct stenosis on preoperative imaging examinations; (3) No suspicious malignant liver-occupying sites; and (4) No obvious jaundice or cholangitis.

In recent years, newer interventional therapies such as percutaneous transhepatic choledochoscopic lithotripsy (PTCSL) have been attempted by an increasing number of surgeons. Since its development in the 1970s[20], PTCSL has undergone major advancements and shows an ideal effect when combined with 3D visualization techno-logy[21,22]. One study reported that PTCSL could be performed in patients with biliary strictures and yielded an optimal effect[23]. However, since most hepatobiliary surgeons have not gained expertise in this new technique, patients undergoing PTCSL were not included in this study for comparison. Through continuous learning, we hope to conduct prospective studies in the future to verify and enrich the pre-diction model of the nomogram in our study.

Table 3 Univariable and multivariable logistic regression analyses of the risk factors for a poor prognosis in patients with recurrent hepatolithiasis

Variable	Univariable analysis		Multivariable analysis	
	P value	OR (95%CI)	*P* value	OR (95%CI)
Sex: Female/Male	0.328	1.311 (0.762-2.257)		
Age (yr): ≥ 60/< 60	0.603	1.139 (0.697-1.862)		
BMI	0.068	0.921 (0.843-1.006		
Abdominal pain: Yes/No	0.066	0.525 (0.264-1.043)		
Fever: Yes/No	0.083	1.551 (0.945-2.547)		
Jaundice: Yes/No	0.406	1.270 (0.724-2.230)		
Previous operation times				
2 times/1 time	0.384	1.337 (0.695-2.571)	0.299	1.451 (0.719-2.932)
3 times/1 time	< 0.001	4.840 (2.241-10.454)	< 0.001	4.573 (2.015-10.378)
≥ 4 times/1 time	0.005	7.340 (1.827-29.498)	0.018	5.741 (1.347-24.470)
Previous hepatectomy: Yes/No	0.026	1.936 (1.082-3.463)	0.144	1.642 (0.845-3.190)
Previous cholangioenterostomy: Yes/No	0.455	1.299 (0.654-2.577)		
NLR: ≥ 2.462/< 2.462	0.001	2.334 (1.410-3.863)	0.022	1.915 (1.099-3.337)
PLR: ≥ 173.74/< 173.74	0.069	1.714 (0.959-3.065)		
AGR: ≤ 1.5/> 1.5	0.002	2.459 (1.393-4.338)	0.033	1.949 (1.056-3.595)
TB (μmol/L): ≥ 34.2/< 34.2	0.381	1.330 (0.703-2.518)		
ALT (IU/L): ≥ 50/< 50	0.664	1.117 (0.677-1.843)		
AST (IU/L): ≥ 40/< 40	0.169	1.418 (0.862-2.333)		
ALP (IU/L): ≥ 200/< 200	0.060	1.613 (0.981-2.654)		
GGT (IU/L): ≥ 150/< 150	0.464	1.202 (0.735-1.967)		
HBsAg: Positive/Negative	0.791	0.890 (0.377-2.103)		
CA19-9 (U/mL): ≥ 34/< 34	0.058	1.656 (0.984-2.787)		
Operation duration (h)				
2-4/≤ 2	0.803	1.085 (0.572-2.057)		
> 4/≤ 2	0.497	0.794 (0.408-1.545)		
Liver cirrhosis: Yes/No	0.049	2.008 (1.004-4.016)	0.478	1.343 (0.595-3.034)
Hepatic atrophy: Yes/No	0.469	0.833 (0.507-1.368)		
Intrahepatic stenosis: Yes/No	0.054	1.772 (0.989-3.176)		
Extrahepatic stones: Yes/No	0.481	0.810 (0.450-1.456)		
Hepatectomy: Yes/No	0.019	0.543 (0.326-0.904)	0.211	0.692 (0.389-1.232)
Bilateral hepatolithiasis: Yes/No	0.011	2.114 (1.183-3.775)	0.038	1.965 (1.039-3.717)
Drainage mode				
Cholangioenterostomy/External T tube drainage	0.292	0.663 (0.308-1.425)		
Combined drainage/External T tube drainage	0.325	1.381 (0.726-2.629)		
Function of the SO				
Dysfunction/Normal	0.760	1.110 (0.567-2.173)		
Nonfunctional/Normal	0.051	1.791 (0.997-3.219)		
Resected/Normal	0.845	0.905 (0.332-2.464)		
Intraoperative bleeding (mL): ≥ 400/< 400	0.682	1.264 (0.412-3.883)		

Blood transfusion: Yes/No	0.053	1.946 (0.993-3.815)		
TB after operation (μmol/L): ≥ 34.2/< 34.2	0.908	1.035 (0.576-1.862)		
Bile culture: Positive/Negative	0.384	1.255 (0.753-2.093)		
Clavien-Dindo classification: ≥ III/< III	0.541	1.430 (0.455-4.494)		
Hospitalization expenses	0.913	1.000 (1.000-1.000)		
Immediate clearance: No/Yes	< 0.001	3.271 (1.874-5.711)	0.005	2.398 (1.304-4.409)
Final clearance: No/Yes	0.030	3.098 (1.117-8.595)	0.558	1.448 (0.420-4.996)

BMI: Body mass index; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; AGR: Albumin-to-globulin ratio; TB: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; HbsAg: Hepatitis B surface antigen; CA19-9: Carbohydrate antigen19-9; SO: Sphincter of Oddi; OR: Odds ratio; CI: Confidence interval.

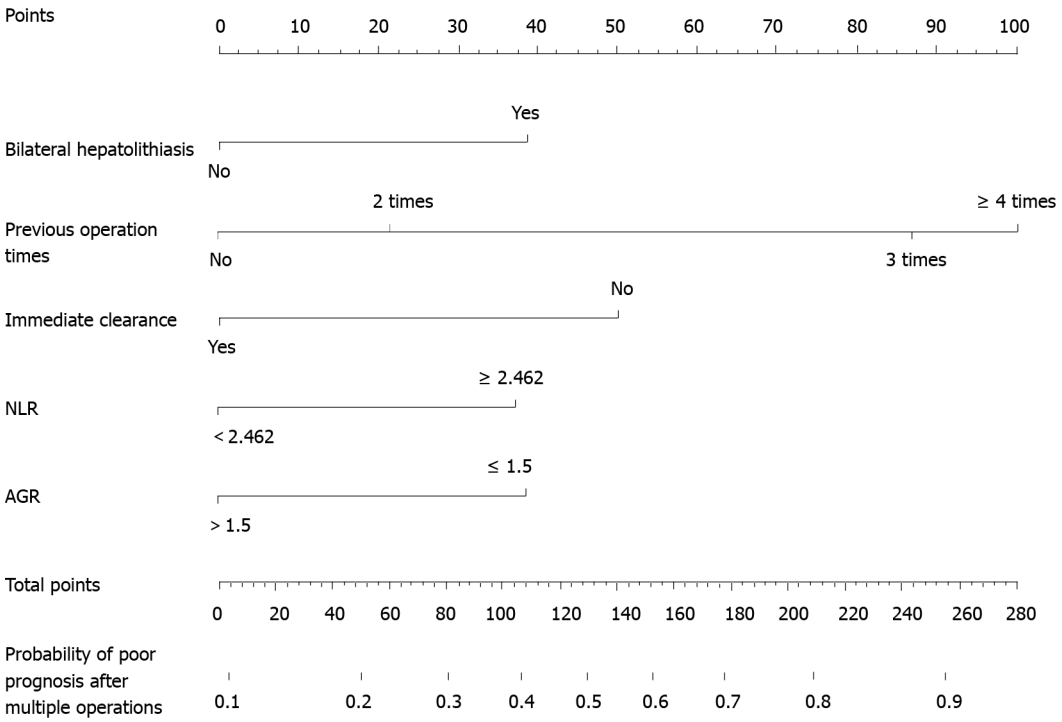


Figure 1 Nomogram for predicting prognosis in the training cohort. NLR: Neutrophil-to-lymphocyte ratio; AGR: Albumin-to-globulin ratio.

The treatment of bilateral hepatolithiasis is more complicated and difficult than that of unilateral hepatolithiasis: Intraoperative lithotomy is more difficult, and the postoperative residual stone rate is higher[19]. Moreover, even if liver resection is performed, it is difficult to avoid residual stones on the opposite side of the liver, resulting in a higher probability of poor prognosis or recurrence. Many studies have confirmed these outcomes[18,24,25]. Hepatectomy on the severe side combined with choledochoscopic lithotripsy is a better treatment for bilateral hepatolithiasis with or without intrahepatic biliary strictures[18,19]. Due to the repeated stone stimulation and attacks of cholangitis, the affected hepatic segments are usually damaged, atrophied, or narrowed, while the unaffected hepatic segments may show compen-satory hyperplasia. Anatomical hepatectomy is a crucial factor in the treatment of hepatolithiasis[18,26,27]. In addition, for bilateral hepatolithiasis, the use of three-dimensional reconstruction has been shown to improve the immediate clearance rate (96.1% *vs* 81%) and the final clearance rate (100% *vs* 90.5%)[28].

The nomogram also included two laboratory indicators, NLR and AGR. As common indicators of immune function and inflammation, NLR and AGR have been used to determine the prognosis of various benign and malignant diseases[29-32]. AGR reflects the degree of inflammation as well as the nutritional status of the human body, which can form a vicious cycle and promote disease development [33]. A reduction or inversion of AGR, which also appears in cirrhosis and chronic hepatitis, indicates serious liver damage. A previous study indicated that elevated NLR is an independent risk factor for secondary intrahepatic cholangiocarcinoma (ICC) after surgery for hepatolithiasis[34], but the association between elevated NLR and ICC remains to be elucidated. Inflammation and subtle

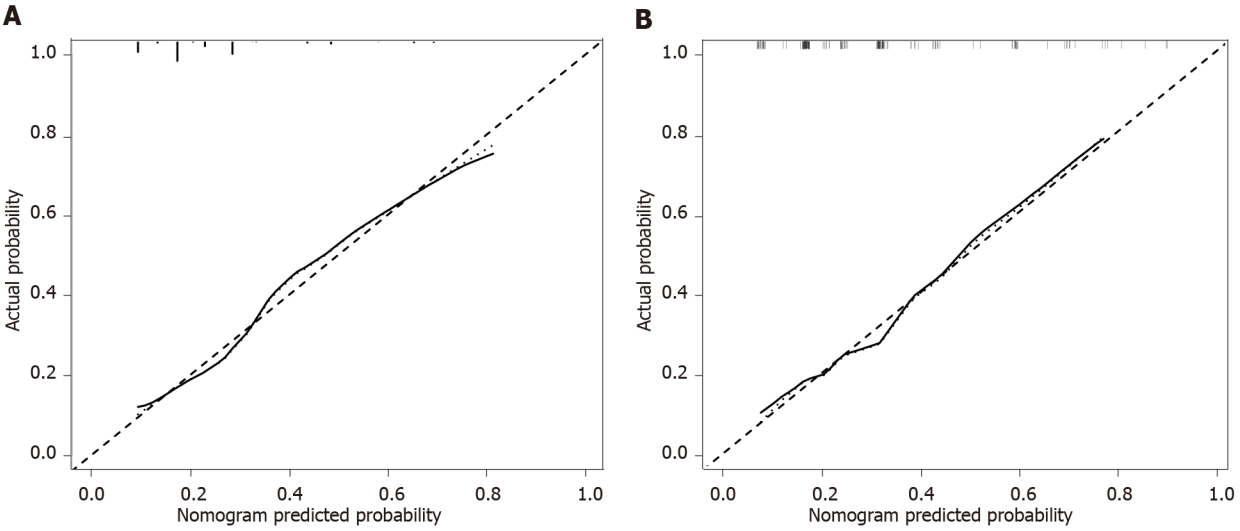


Figure 2 Calibration curves for predicting the prognosis. The nomogram had c-index values of 0.748 and 0.743 in the training and validation cohorts, respectively. A: In the training; B: In the validation cohorts.

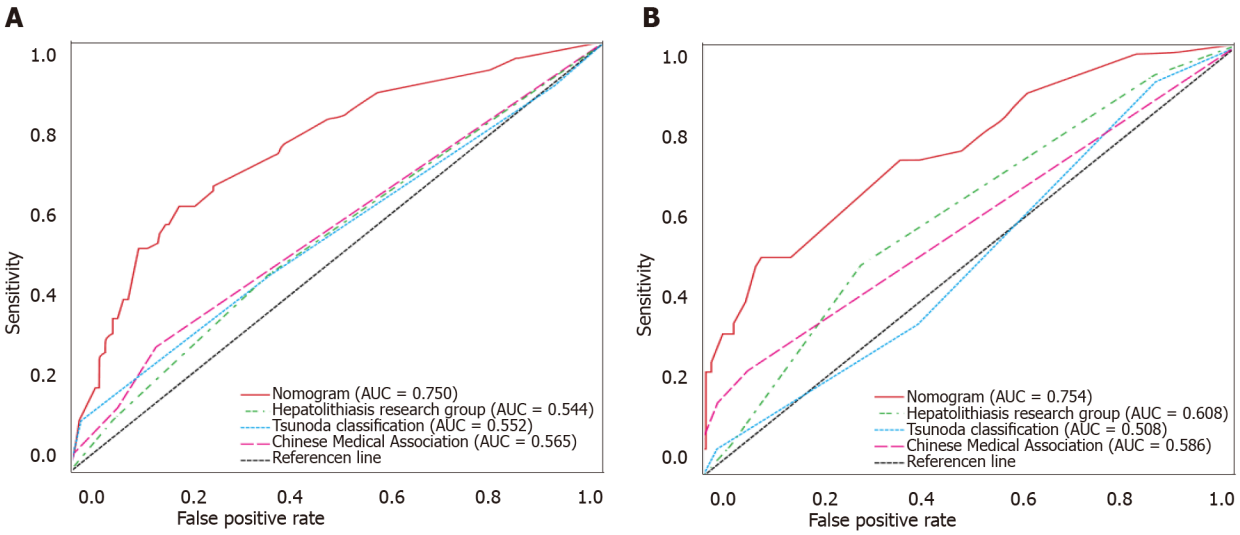


Figure 3 Receiver operating characteristic curve of our nomogram and other three traditional classifications. A: In the training; B: In the validation cohorts. AUC: Area under the curve.

alterations in immune regulation may play important roles in this process[35]. We found that NLR and AGR independently affected the prognosis of patients with recurrent hepatolithiasis. Therefore, we included both in the nomogram and validated them in the validation group.

This study highlights the problem of concomitant ICC, which has been reported to occur in 2.5% of patients with intrahepatic stones[19]. To avoid the influence of malignant tumors on the prognosis of patients with hepatolithiasis during the follow-up period, patients in the training and validation groups who showed ICC at the time of hospitalization were excluded (7.7%). Moreover, cancer was regarded as one of the long-term complications of hepatolithiasis in the follow-up, and was classified as a follow-up endpoint (15 patients, 3.4%), in agreement with previous studies[19,25,34,36,37].

Our study has some limitations that merit discussion. First, although laparoscopic treatment of biliary tract stones has gradually attracted research attention, some studies have suggested that laparoscopic treatment of biliary tract stones in patients with a history of biliary tract surgery is feasible and has advantages[38,39]. In our study, only 35 and 19 patients in the training and validation groups, respectively, were completely operated by laparoscopy. We did not use laparoscopic surgery as a routine procedure because all patients included in this study had a history of biliary tract surgery, unlike the previous studies. During the actual surgeries, the abdominal cavity adhesions of patients with different surgical durations were very different. Converse-ly, a previous study reported that laparoscopic surgery and open surgery for patients with a history of biliary system surgery showed no statistically significant differences in the surgical duration, blood loss, the postoperative hospitalization

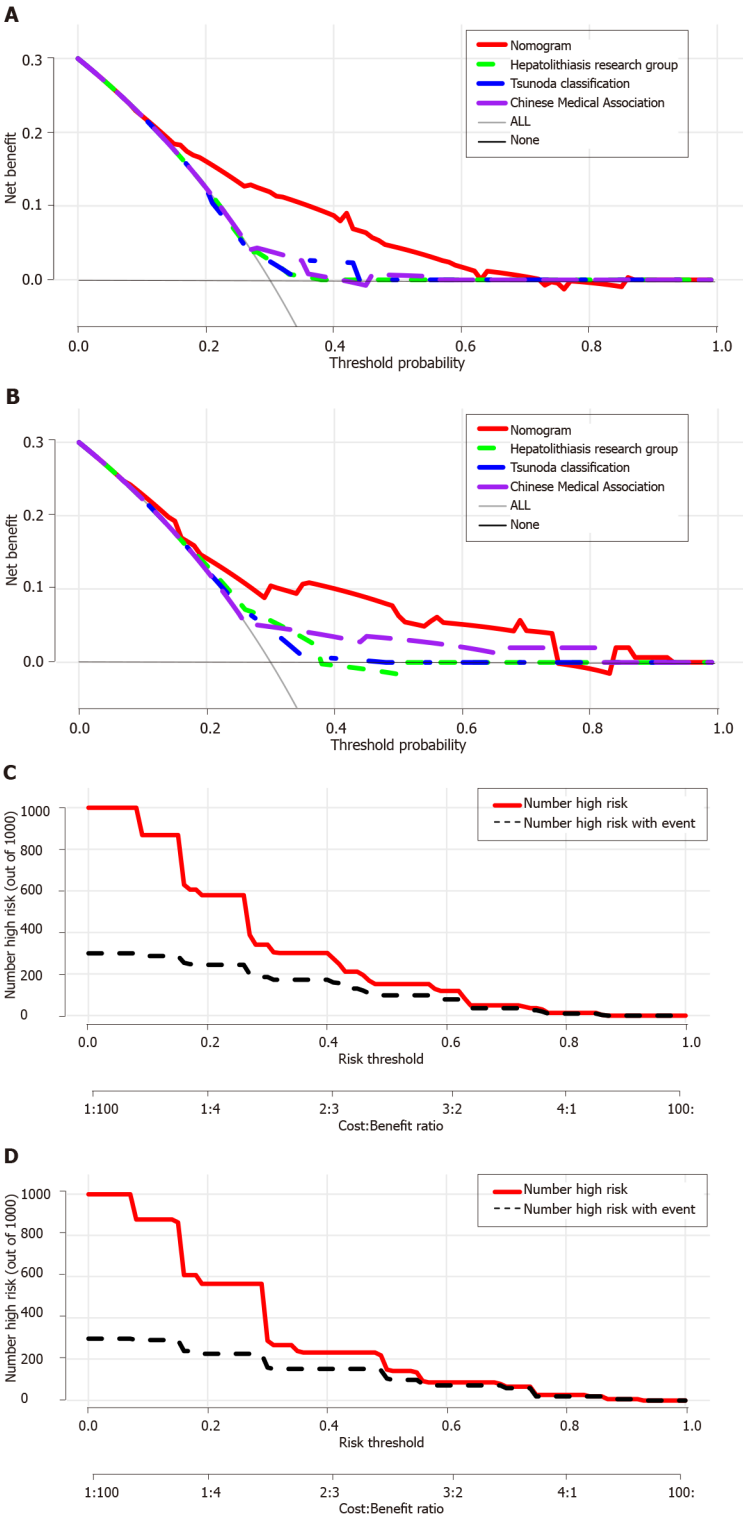


Figure 4 The decision curve analysis of our nomogram and three other traditional classifications. The x-axis represents the threshold probability and the y-axis represents the net benefit. The horizontal solid black line represents the hypothesis that no patients reached the endpoint, and the solid gray line represents the hypothesis that all patients met the endpoint. A: In the training; B: In the validation cohorts; C: Clinical impact curves of the nomogram in the training; D: Validation cohorts. At different threshold probabilities within a given population, the number of high-risk patients and the number of high-risk patients with the outcome are shown.

duration, postoperative complications, and the calculi clearance rate[40]. Moreover, considering the majority of rural patients, economic affordability also needs to be considered. Thus, since many patients had undergone multiple biliary tract surgeries, we chose the most suitable surgical mode according to the individual patient characteristics.

Moreover, this was a retrospective study with inherent defects as a result of potential biases, and prospective validation is required to confirm the value of the findings. Since the aim of this study was to establish prognosis prediction in surgically treated recurrent hepatolithiasis patients, subsequent

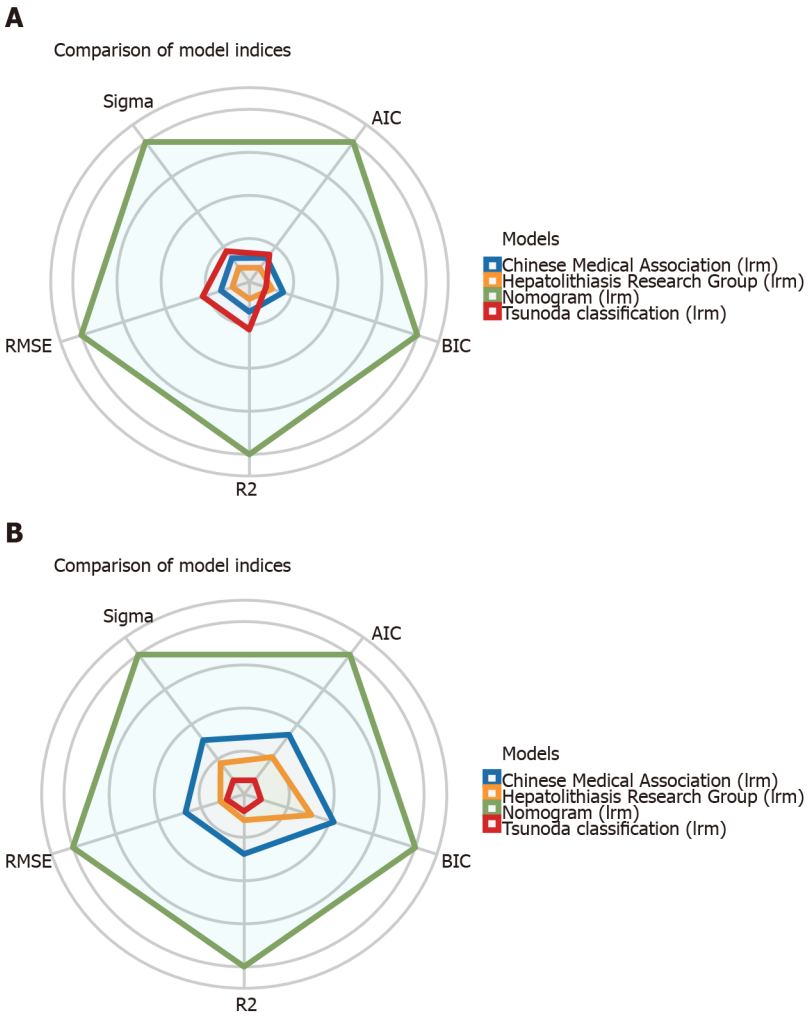


Figure 5 Visual fit indices of our nomogram and other three traditional classifications. A: In the training; B: In the validation cohorts.

treatment and prognosis of patients who progressed to ICC after reoperation were not analyzed further. Lastly, the present algorithm considered only patients who underwent surgery; therefore, a selection bias is likely.

CONCLUSION

In conclusion, our study is the first to develop and validate a novel online nomogram based on independent risk factors to dynamically predict the prognosis of patients with recurrent hepatolithiasis after reoperation. The nomogram is easy to use, highly accurate, and shows excellent calibration. The nomogram demonstrated superior performance and discriminative power compared to the three traditional classifications, which can help clinicians alert people at a higher risk of poor prognosis as early as possible and provide information for designing personalized clinical treatment of different patients.

ARTICLE HIGHLIGHTS

Research background

Hepatolithiasis is a refractory benign disease with high recurrence rate. Many patients have poor prognosis.

Research motivation

There have been no large studies of patients with hepatolithiasis, and there are no clear risk factors for prognosis in these patients.

Research objectives

We aimed to find the risk factors affecting the prognosis of these patients and establish a prediction model which is conducive to clinical surgical decision-making.

Research methods

We collected data of hepatolithiasis patients in four large medical centers, identified independent risk factors and established nomogram. And then we used the concor-dance index, calibration, area under curve, decision curve analysis, clinical impact curves, and visual fit indices to evaluate the accuracy of the nomogram.

Research results

Multiple previous surgeries, bilateral hepatolithiasis, absence of immediate clearance, neutrophil-to-lymphocyte ratio ≥ 2.462, and albumin-to-globulin ratio ≤ 1.5 were found to be independent factors influencing the prognosis. And our nomogram has a higher predictive value than traditional classifications.

Research conclusions

A nomogram for predicting the prognosis of patients with recurrent hepatolithiasis was established for the first time, and an online calculator was set up to help surgeons make clinical decisions.

Research perspectives

More medical centers included, more data collection, and application of "Artificial Intelligence".

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FOOTNOTES

Author contributions: Pu T, Chen JM, Li ZH, and Jiang D contributed to the data analysis and participated in drafting the article; Guo Q, Li AQ, and Cai M extracted the clinical data and calculated the clinical correlations; Chen ZX, Xie K and Zhao YJ interpreted the results and revised the manuscript; Liu FB, Hou H, Lu Z, Wang C, and Geng XP gave final approval of the version to be published; and all authors contributed to the design and interpretation of the study and to further drafts and approved the final version to be published.

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Country/Territory of origin: China

ORCID number: Tian Pu 0000-0002-6961-0814; Jiang-Ming Chen 0000-0003-4683-5866; Zi-Han Li 0000-0003-0378-0252; Dong Jiang 0000-0003-3372-6062; Qi Guo 0000-0002-3808-0224; Ang-Qing Li 0000-0002-5156-1056; Ming Cai 0000-0003-0001-0316; Zi-Xiang Chen 0000-0002-5782-2625; Kun Xie 0000-0002-2491-4988; Yi-Jun Zhao 0000-0001-9082-8861; Cheng

Wang 0000-0001-7899-2079; Hui Hou 0000-0002-5997-1229; Zheng Lu 0000-0001-7249-9961; Xiao-Ping Geng 0000-0002-6219-6366; Fu-Bao Liu 0000-0003-3431-2187.

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ORIGINAL ARTICLE

Observational Study

Effect of *Bifidobacterium longum* 35624 on disease severity and quality of life in patients with irritable bowel syndrome

Jean-Marc Sabaté, Franck Iglicki

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Jean-Marc Sabaté, Department of Gastroenterology, Hôpital Avicenne, AP-HP, Sorbonne Paris Nord, Bobigny 93000, France

Jean-Marc Sabaté, INSERM U-987, Pathophysiology and Clinical Pharmacology of Pain, Ambroise Paré Hospital, Boulogne-Billancourt 92100, France

Franck Iglicki, 15 allée Léon Gambetta, Clichy 92110, France

Corresponding author: Jean-Marc Sabaté, MD, PhD, Full Professor, Department of Gastroenterology, Hôpital Avicenne, AP-HP, Sorbonne Paris Nord, 125 rue de Stalingrad, Bobigny 93000, France. jean-marc.sabate@aphp.fr

Abstract

BACKGROUND

Bifidobacterium longum 35624 has shown efficacy in improving irritable bowel syndrome (IBS) symptoms compared with placebo in double-blind randomized studies. However, few data are available from real-life clinical practice or from studies that used Rome IV criteria to diagnose IBS.

AIM

To assess the effect of *B. longum* 35624 on IBS severity and quality of life in a real-life setting.

METHODS

From November 2018 to January 2020, 278 patients with IBS (according to Rome IV criteria) were enrolled in a prospective, open-label, multicenter observational study by private practice gastroenterologists to received one capsule of *B. longum* 35624 (10^9 colony-forming units) *per* day for 30 d. Participation in the study was independently proposed to patients during spontaneous consultations. Disease severity (assessed by the IBS severity scoring system) and patient quality of life (assessed by the IBS quality of life questionnaire) were compared between the inclusion visit (baseline) and the visit at the end of 30 d of treatment. The characteristics of patients were described at baseline. Continuous variables comparisons between inclusion and end-of-treatment visits were performed using the t-test and Kruskal-Wallis test. Categorical variables comparisons were performed using the $\chi 2$ test.

RESULTS

A total of 233 patients, with a mean age of 51.4 years and composed of 71.2% women, were included in the study. Of these patients, 48.1% had moderate IBS and 46.4% had severe IBS. After a 30-d treatment period with one *B. longum* 35624 capsule *per* day, a significant decrease in IBS severity was observed compared to baseline (mean ± SD, IBS severity scoring system scores: 208 ± 104 *vs* 303 ± 81, $P < 0.001$) and 57% of patients moved to lower severity categories or achieved remission. The quality of life of patients was also improved by the treatment (IBS Quality of Life questionnaire score: 68.8 ± 20.9 *vs* 60.2 ± 20.5; $P < 0.001$) and 63.8% of patients were satisfied with the treatment.

CONCLUSION

Thirty days of treatment with *B. longum* 35624 reduces disease severity and improves the quality of life of patients with IBS, particularly those with the most severe forms of IBS.

Key Words: Irritable bowel syndrome; Probiotics; *Bifidobacterium longum*; Quality of life; Severity of illness index; Abdominal pain

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Core tip: Our observational study of 233 patients with moderate-to-severe irritable bowel syndrome demonstrated that 30 d of treatment with once-daily *Bifidobacterium longum* 35624 (a probiotic) resulted in a significant improvement in symptoms and disease severity for two thirds of the patients. Patient quality of life was also significantly improved, and the majority of patients expressed that they were satisfied with the treatment.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional bowel disorder, which combines abdominal pain and transit disorders and affects approximately 4% to 10% of the population, depending on whether the Rome III or Rome IV definition is used[1]. IBS may be responsible for impairment in quality of life, especially in the most severe forms, which represent approximately 25% of cases[2]. The pathophysiology of IBS is complex and multifactorial, involving both peripheral and central factors[3]. Several lines of evidence suggest that the intestinal microbiota plays a role in the pathophysiology of IBS, including the existence of post-infectious forms of IBS, and the difference in microbiota composition between patients with IBS and healthy individuals. Moreover, studies carried out in humans and in animal models have shown that strategies targeting the gut microbiota are effective in the treatment of IBS[4].

Probiotics are available over the counter and few have real recommendations from learned societies and/or claims substantiated by findings from properly conducted clinical trials. Few probiotics have shown efficacy in improving IBS symptoms relative to placebo in randomized double-blind studies[5], as it is the case with *Bifidobacterium longum* 35624 (formerly *B. infantis*) with two conclusive randomized clinical trials[6,7]. Studies of probiotics have been criticized for the criteria used to measure efficacy[8]. Few studies have thus far adopted the efficacy criteria for the assessment of treatments for IBS specified by the United States Food and Drug Administration or the European Medicines Agency (EMA)[9-11]. At a minimum, studies should demonstrate the ability of a probiotic to reduce disease severity, as is the case for fecal microbiota transplant studies[12], and to improve quality of life. However, in most clinical studies of probiotics, disease severity at baseline is often not reported, patients with mild or moderate forms of IBS are recruited and quality of life is not evaluated. The strengthening of the diagnostic criteria for IBS, with the deletion of the term "dis-comfort" and the modification of the frequency of abdominal pain that patients must present in the new version of the Rome criteria (the Rome IV criteria), had the effect of decreasing the incidence of the disease and increasing the percentage of severe forms among patients[13]. Few studies have assessed the efficacy of probiotics in the Rome IV era.

The aim of our study was to assess the effect of *B. longum* 35624 treatment on IBS severity and patients' quality of life in real-life clinical practice.

MATERIALS AND METHODS

Study design and population

This was a prospective, open-label, multicenter study (FLORAVIE study). Patients over 18 years of age with IBS diagnosed according to the Rome IV criteria were enrolled from November 2018 to January 2020. Patients had to have had recurrent abdominal pain on average at least 1 d/wk in the last 3 mo, associated with two or more of the following criteria: Pain related to defecation or associated with a change in frequency of stool or associated with a change in form (appearance) of stool. IBS diagnosis criteria had to be fulfilled for the last 3 mo with symptom onset at least 6 mo prior to diagnosis. Participation in the study was proposed by private gastroenterologists during spontaneous consultations with new or former patients consulting for their IBS. The gastroenterologist was free to prescribe or not *B. longum 35624* according to his appreciation if he tought it was a good therapeutic option for the patient. Participating patients were seen in consultation at a second visit after 30 d of treatment.

This study was conducted according to the guidelines of the Declaration of Helsinki and the Guidelines for Good Clinical Practice (EMA/Committee for Medicinal Products for Human Use/International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use/135/1995) and approved by the North-west III Protection of Persons Committee of the University Hospital of Caen, France. Study conduct was in full accordance with French applicable laws and regulations, including but not limited to current International Council on Harmonisation-Good Clinical Practices. All participants received an information note and were informed about the objectives, methodology and purpose of the study, and those who agreed to participate were required to provide oral non-opposition prior to entry, according to France regulation. The study was registered on clinicaltrials.gov under study number NCT04662502. To ensure that the patient population was representative of the country, participants were recruited by gastroenterologists working in private practice (with a maximum of 12 patients *per* investigator) throughout France.

Eligible patients were included only if they met all the inclusion and none of the exclusion criteria. Previous treatment with *B. longum* 35624 was an exclusion criterion. The use of other probiotics was not allowed within 2 wk from inclusion. Recent antibiotic treatment or treatment modification that could have an impact on microbiota, gut motility or digestive symptoms were prohibited. Participants with a history of abdominal surgery (except for appendectomy, cholecystectomy, surgery for hemorrhoids or cesarean section) were excluded.

After obtaining the patient's oral non-opposition to the study and verifying the inclusion and exclusion criteria, treatment with *B. longum* 35624 was initiated. Patients who were included in the study were to take one capsule *per* day containing 10^9 colony-forming units of *B. longum* 35624 for 30 d. Patients were advised not to change their diet during the study. Clear instructions were provided to patients and gastroenterologists on how to complete and review the study questionnaires.

During the first visit, patients' demographics (sex, age), medical history, medical conditions other than IBS and concomitant treatments, disease characteristics (mode of onset, disease duration, transit sub-type, treatments, associated conditions) and the effect of IBS on their personal, professional, and social life were collected. The effect of IBS on the patient's personal, professional, and social life was assessed using a six-point Likert scale, ranging from "no impact" to "very severe impact". Patients recorded their treatment intake in a daily diary. Stool consistency was assessed using the Bristol stool scale[14] at baseline and every 10 d throughout the study and recorded in the diary. The severity of IBS was determined using the IBS severity scoring system (IBS-SSS), which consists of five domains assessing the intensity and frequency of abdominal pain, intensity of abdominal distension, satisfaction with transit and quality of life. IBS-SSS scores range from 0 to 500, with scores < 75 indicating remission, scores between 75 and 174 indicating mild severity, scores between 175 and 299 indicating moderate severity, and scores between 300 and 500 indicating severe disease[15]. Quality of life was assessed using the IBS quality of life questionnaire (IBS-QOL), which consists of 34 questions exploring eight dimensions, including dysphoria, interference with activity, body image, health worry, food avoidance, social reaction, sexual and relationships, with scores ranging from 0 to 100, 100 indicating the best quality of life[16]. IBS-SSS and IBS-QOL questionnaires were administered at baseline before treatment and after 30 d of treatment with *B. longum* 35624 (second visit). Satisfaction with the treatment was assessed by gastroenterologists and by patients independently using a five-point Likert scale. Adherence to treatment was assessed by recording daily medication intake in the patient's diary and based on the gastroenterologist's records at the follow-up visit. Adverse events (AEs) occurring throughout the study were recorded, as were any events that could have interfered with *B. longum* 35624 treatment, including changes in diet.

The main outcomes were the proportion of patients who had a decrease of > 50 points in the IBS-SSS score and the proportion of patients who had an increase of > 10 points in the IBS-QOL score after a 30-d treatment with *B. longum* 35624. Secondary outcomes were the change in IBS-SSS and IBS-QOL scores, the proportion of patients who had a shift from one severity category to another, and the proportion of patients and gastroenterologists who were satisfied with the treatment.

Considering a variation in the IBS-QOL score of 15, the total number of patients to be assessed was determined to be 203 to obtain a level of precision of 10%. To maintain this level of precision considering that a certain number of patients (about 10%) would not be eligible for primary endpoint analysis (missing data), 220 patients had to be included in the study.

The safety population included all patients who received at least one dose of *B. longum* 35624. The evaluable population included all patients in the safety population with at least one post-baseline assessment who did not use any drugs that could have interfered with effect of *B. longum* 35624.

Statistical methods and data analysis

The statistical methods of this study were reviewed by a biomedical statistician from ICTA (International Clinical Trials Association, fontaine-les-Dijon, France). Statistical analyses were performed using SAS® software (version 9.2-SAS Institute, North Carolina, United States). Means and SD or medians and interquartile ranges (IQR) were calculated for continuous variables and comparisons were performed using the *t*-test and Kruskal-Wallis test. Frequencies were calculated for categorical variables and comparisons were performed using the χ^2 test. Comparisons of IBS-QOL or IBS-SSS scores at baseline *vs* at the end of treatment were performed using the Wilcoxon test. Correlations between variables were evaluated using the Pearson correlation coefficient. Missing data were not replaced.

RESULTS

This observational study was proposed to 129 gastroenterologists throughout France, of whom, 86 were interested in participating and 61 recruited patients. They were representative of the profession with 80% being male (*vs* 70% nationally) and distributed all over the territory, in 12 of the 13 French regions, to be representative of the national practice (Supplementary Figure 1).

From November 2018 to January 2020, 278 patients were enrolled in the study, of which 233 were included in the evaluable population. The detailed flowchart of the population is shown in Figure 1.

Baseline data

Patient baseline characteristics are presented in Table 1. The patient cohort consisted primarily of middle-aged women, most were non-smokers (87.7%) and did not consume alcohol (63.9%). Body mass index was classified as normal in 51.2% of patients, as overweight in 33.5%, as obese in 9.4%, and as underweight in 5.2%. Thirty-two percent of patients (n = 75) had at least one pre-existing condition, the most common of which were gastrointestinal conditions other than IBS (7.3%, n = 17; gastroesophageal reflux, hiatal hernia, colonic diverticulum, hemorrhoids), endocrine (5.2%, n = 12) and vascular disorders (5.2%, n = 12). In addition, 21.0% of patients received at least one concomitant treatment that was unlikely to interfere with study treatment. At baseline, thirty-seven patients (15.8%) had already been on a specific diet [low fermentable oligo-, di-, mono-saccharides and polyols (FODMAP) diet, n = 7; gluten-free diet, n = 2; lactose-free diet, n = 4] for more than 1 mo, and did not change it during the study period.

IBS was triggered by an acute episode of gastroenteritis (post-infectious IBS) in nine patients (3.9%) and by a stressful event in 91 patients (34.8%), with sexual abuse reported in eight patients. No triggering factor for IBS was identified in 127 patients (54.5%). Median disease duration was 8.0 years (IQR: 3.0, 16.0). Diarrhea-predominant IBS (IBS-D) (38.2%) was the most common subtype, while IBS of unidentified subtype (IBS-U; 5.2%) was the least common; antispasmodics and transit modifiers had previously been prescribed respectively in 65.7% and 35.7% of IBS patients.

At baseline, the average IBS severity among the included patients was high (mean ± SD IBS-SSS score 303.0 ± 81.5), with the majority having either a severe or a moderate form of the disease, and fewer than 10% having mild disease severity or being in remission. IBS severity scores were different across transit pattern subtypes (Figure 2A) with constipation-predominant (IBS-C) and IBS-D having the highest scores (326.8 ± 84.2 and 300.8 ± 81.2, respectively) compared to mixed IBS (IBS-M) and IBS-U (284.1 ± 77.9 and 277.8 ± 54.3, respectively).

Quality of life was impaired in most patients (mean ± SD IBS-QOL score 60.2 ± 20.5). Quality of life scores were comparable across transit pattern subtypes (Figure 2B) and were correlated with disease severity (r = −0.66, P < 0.0001), with higher IBS severity associated with lower the IBS-QOL scores (*i.e.*, lower quality of life) (Figure 3). In 96% of cases, IBS impaired the patient's personal, professional, and social life (low or mild in 26.0% of patients, moderate in 37.7%, and severe or very severe in 36.3% of patients).

Follow-up data

Duration of exposure to study treatment was 28.2 ± 3.4 d (mean ± SD). Patients' adherence to treatment according to patients' daily diary reports was excellent, with 94.1% adherence to capsule intake.

After 30 d of treatment, there was a significant reduction in IBS severity compared with baseline (mean ± SD overall IBS-SSS score: 208.1 ± 104.8 *vs* 303.2 ± 81.5, P < 0.001) (Figure 4A), and in all transit subtypes (Figure 2A). The evolution of IBS severity scores was similar and of the same magnitude in all

Table 1 Baseline characteristics of the study participants (mean ± SD)

Characteristic	IBS group, *n* = 233
Median age (IQR), yr	51.0 (37–66)
Female sex, *n* (%)	166 (71.2)
BMI (kg/m^2)	24.5 ± 4.3
IBS sub-types (transit pattern), *n* (%)	
IBS-C	71 (30.5)
IBS-D	89 (38.2)
IBS-M	61 (26.2)
IBS-U	12 (5.2)
IBS severity categories (IBS-SSS), *n* (%)	
Remission (0–74)	2 (0.8)
Mild (75–174)	11 (4.7)
Moderate (175–299)	112 (48.1)
Severe (> 300)	108 (46.4)
IBS-QOL (0–100)	60.2 ± 20.5

BMI: Body mass index; C: Constipation; D: Diarrhea; IBS: Irritable bowel syndrome; IBS-QOL: Irritable bowel syndrome quality of life; IBS-SSS: Irritable bowel syndrome severity scoring system; IQR: Interquartile range; M: Mixed; U: Unclassified.

IBS subtypes (P = 0.115) (Table 2). Hence, after treatment, IBS severity scores were different across transit pattern subtypes (Figure 2A) with IBS-C having the highest scores compared to IBS-D, IBS-M and IBS-U.

Each of the five IBS-SSS domains was improved (Figure 5 and Supplementary Figure 2). In addition, the proportion of patients who reported experiencing symptoms every day over a period of 10 d was reduced by half after treatment, dropping from 76 patients (32.9%) to 34 patients (15.6%). Moreover, the number of pain-free patients over a 10-d period, which was low at baseline (0.4%; one patient), increased to 10.1% (22 patients) after 30 d of treatment.

A change towards categories of lower IBS severity or remission occurred in 56.7% of patients (Figure 6). A significant improvement in disease severity (> 50-point decrease in the IBS-SSS score) was observed in 65.7% of patients, with significant improvement noted in all transit subtypes.

Compared with baseline, the overall quality of life was improved at the end of treatment in the entire patient population (mean ± SD IBS-QOL score 68.8 ± 20.9 *vs* 60.2 ± 20.5; P < 0.001) (Figure 4B) and in IBS-C, IBS-D and IBS-M subgroups (P < 0.001) without difference in absolute score evolution between transit pattern subgroups (P = 0.658) (Table 2 and Figure 2B). A clinically significant meaningful improvement in the overall IBS-QOL score (> 10-point increase) was observed in 36.9% of patients, and was more pronounced in patients with more severe disease at baseline (9.1% of patients with mild IBS, 28.6% of patients with moderate IBS, and 49.1% of patients with severe IBS). Each of the eight dimensions of IBS-QOL were improved at the end of treatment (P < 0.001, Supplementary Figure 3).

Over the course of the study, stool consistency tended to be normalized in all IBS transit subtypes (Figure 7) with a decrease in the percentage of patients reporting stool types 1–2 and 6–7 according to different transit patterns.

Approximately two-thirds of patients (63.8%) and gastroenterologists (63.8%) were satisfied with *B. longum* 35624 treatment at the end of the study.

Safety

During the study, 10 AEs possibly related to the use of *B. longum* 35624 were reported in 4.1% (n = 10) of patients in the safety population (n = 244 patients), including flatulence (n = 3), abdominal pain (n = 2), constipation (n = 1), abdominal distension (n = 1), upper abdominal pain (n = 1), gastrointestinal motor disorder (n = 1), and increased weight (n = 1).

DISCUSSION

While the new Rome criteria have decreased IBS prevalence and increased the percentage of patients

Table 2 Absolute evolution of irritable bowel syndrome quality of life and irritable bowel syndrome severity scoring system after treatment (mean ± SD)

	IBS-C, *n* = 71	IBS-D, *n* = 89	IBS-M, *n* = 61	IBS-U, *n* = 12	*P* values[1]
IBS-SSS scores absolute evolution	-89.5 ± 105.9	-107.4 ± 85.5	-91.7 ± 85.6	-66.0 ± 104.2	0.115 (NS)
IBS-QOL scores absolute evolution	8.9 ± 14.1	10.4 ± 15.8	6.6 ± 14.8	4.7 ± 10.9	0.658 (NS)

[1]Intergroup comparison was assessed with a Kruskal Wallis test.

C: Constipation; D: Diarrhea; IBS: Irritable bowel syndrome; IBS-QOL: Irritable bowel syndrome quality of life; IBS-SSS: Irritable bowel syndrome severity scoring system; M: Mixed; NS: Not significant; SD: Standard deviation; U: Unclassified.

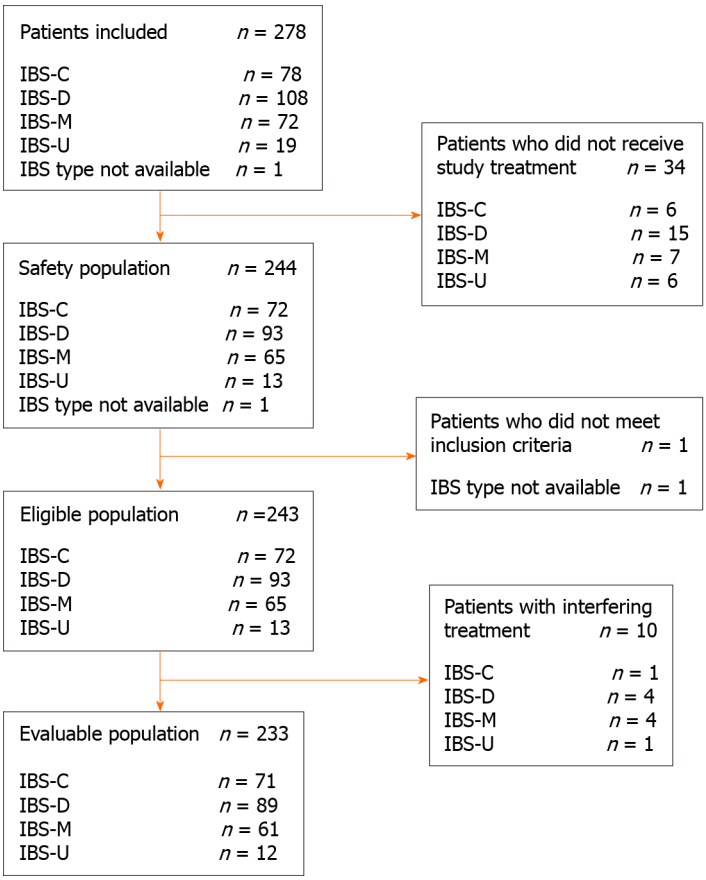


Figure 1 Patient flow. C: Constipation; D: Diarrhea; IBS: Irritable bowel syndrome; M: Mixed; U: "Unclassified" (subtype not determined).

with severe disease, few studies have evaluated the efficacy of probiotics with this new paradigm. In our study conducted in patients with IBS according to Rome IV definition with different transit subtypes and levels of severity, a 30-d treatment regimen with *B. longum* 35624 reduced disease severity and improved quality of life, especially in patients with the most severe forms of IBS.

The significant reduction in disease severity that we found in approximately two-thirds of patients in this study after 30 d of *B. longum* 35624 treatment is consistent with the literature reporting that two-thirds of patients with IBS have dysbiosis, further supporting the link between IBS severity and dysbiosis[17]. It is also consistent with the results of two previous randomized studies of the same probiotic strain, including a large 4-wk study performed in 362 women with IBS[7] and a smaller 8-wk study performed in 80 patients with IBS[6]. The effect on digestive symptoms and disease severity reduction could be secondary to an effect on pro-inflammatory cytokines as it was show previously by O'Mahony *et al*[6]. with a normalisation of an interleukin (IL)-10/IL-12 cytokine ratio that was impaired at baseline[6]. In a recent study[18], at baseline, similar IBS-SSS global scores, IBS severity distribution and IBS subtype distribution were observed for patients with IBS according to Rome IV criteria. Interestingly, in our study, the improvement in overall IBS-SSS score was observed in all its component items (intensity and frequency of abdominal pain, distension and its intensity; satisfaction with bowel habits) and regardless of the IBS subtype (IBS-C, IBS-D, or IBS-M), as also described in the study by Whorwell *et al*[7]. Few studies of probiotics conducted in patients with IBS[19] have included a large

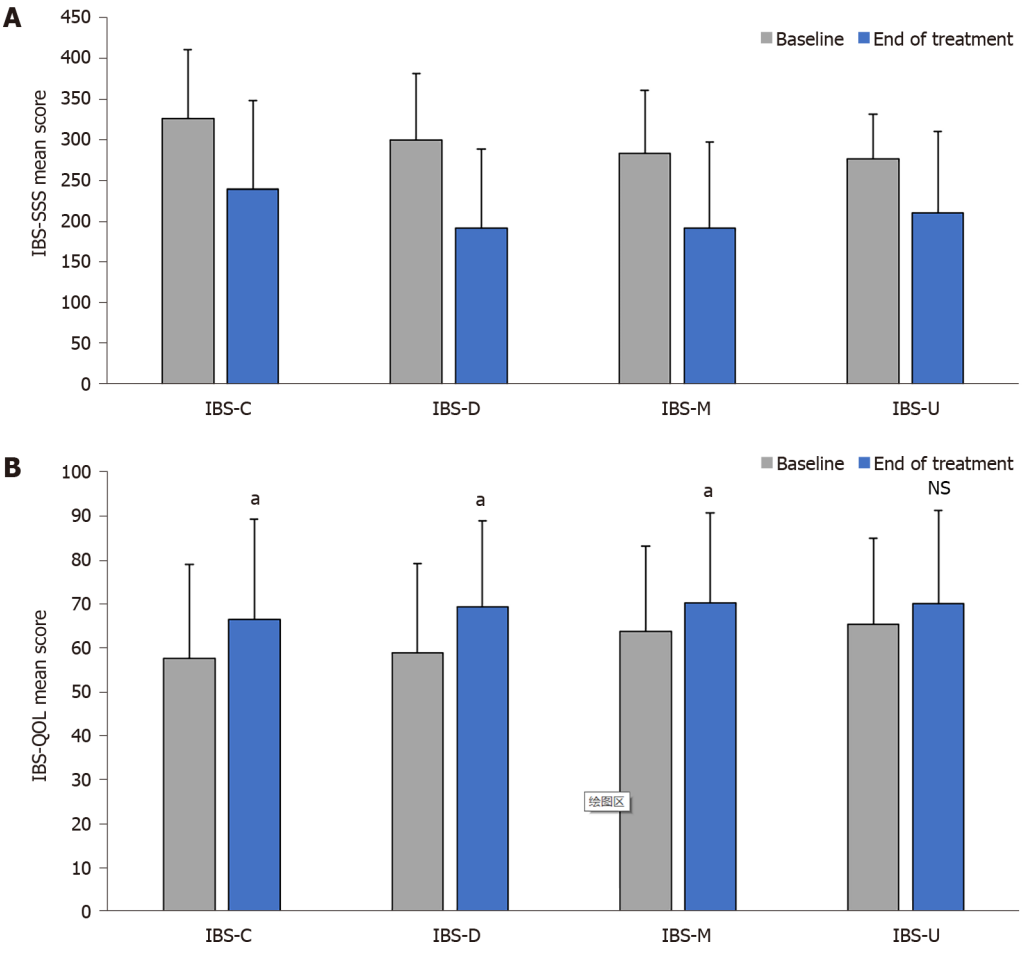


Figure 2 Mean irritable bowel syndrome severity scoring system and Irritable bowel syndrome quality of life score at baseline and at the end of treatment by transit subtype. A: IBS-SSS; B: IBS-QOL. Error bars denote standard deviation. $^{a}P < 0.001$ versus baseline. IBS: Irritable bowel syndrome; IBS-QOL: Irritable bowel syndrome quality of life; IBS-SSS: Irritable bowel syndrome severity scoring system; C: Constipation; D: Diarrhea; M: Mixed; NS: Not significant; U: Unclassified.

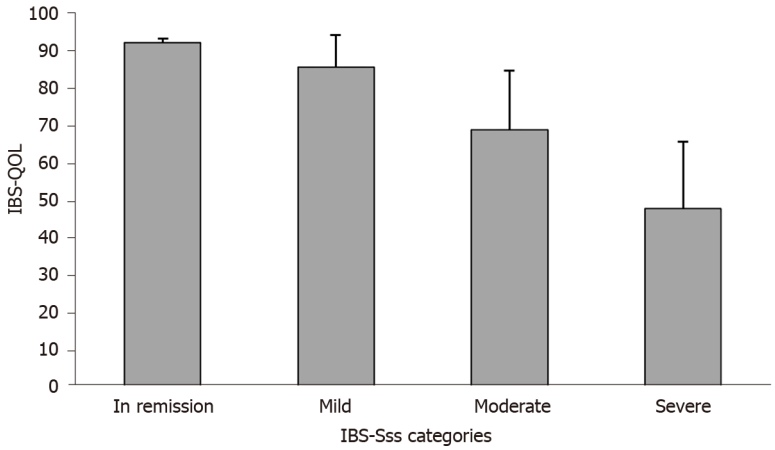


Figure 3 Irritable bowel syndrome quality of life score according to irritable bowel syndrome severity scoring system severity categories at baseline. IBS-QOL: Irritable bowel syndrome quality of life; IBS-SSS: Irritable bowel syndrome severity scoring system.

proportion of patients with severe forms of the disease, as in our present study (approximately half of the patients), and in many such studies, disease severity was not monitored. For a population of patients who are not being treated at tertiary centers, such a level of disease severity may appear surprising; however, a similar level of IBS severity has been reported in patients treated by private gastroenterologists in France[20] using Rome II criteria. Moreover, the selection of patients according to Rome IV criteria tends to increase the proportion of patients with severe IBS[13]. It should be noted that the

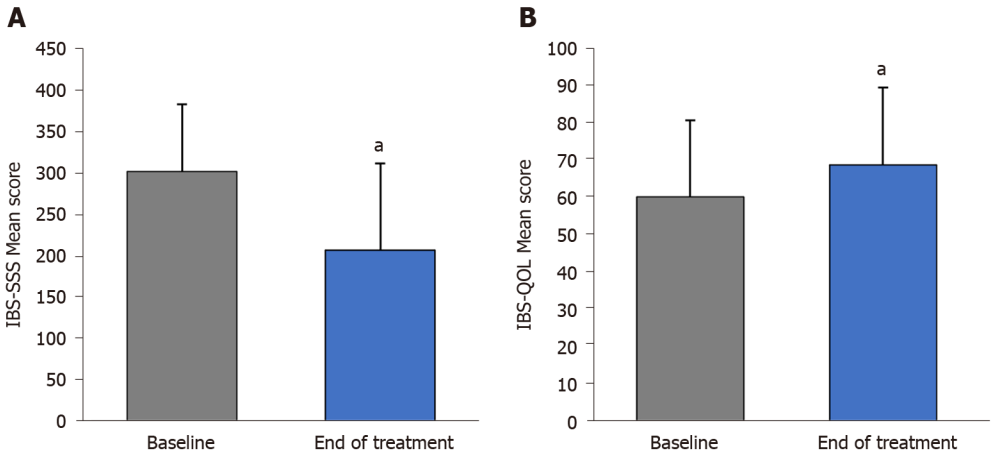


Figure 4 Mean irritable bowel syndrome severity scoring system and irritable bowel syndrome quality of life score at baseline and at the end of treatment. A: Mean irritable bowel syndrome severity scoring system score at baseline and at the end of treatment; B: Mean irritable bowel syndrome quality of life score at baseline and at the end of treatment. Error bars denote standard deviation. [a]*P* < 0.001 *vs* baseline. IBS-QOL: Irritable bowel syndrome quality of life; IBS-SSS: Irritable bowel syndrome severity scoring system.

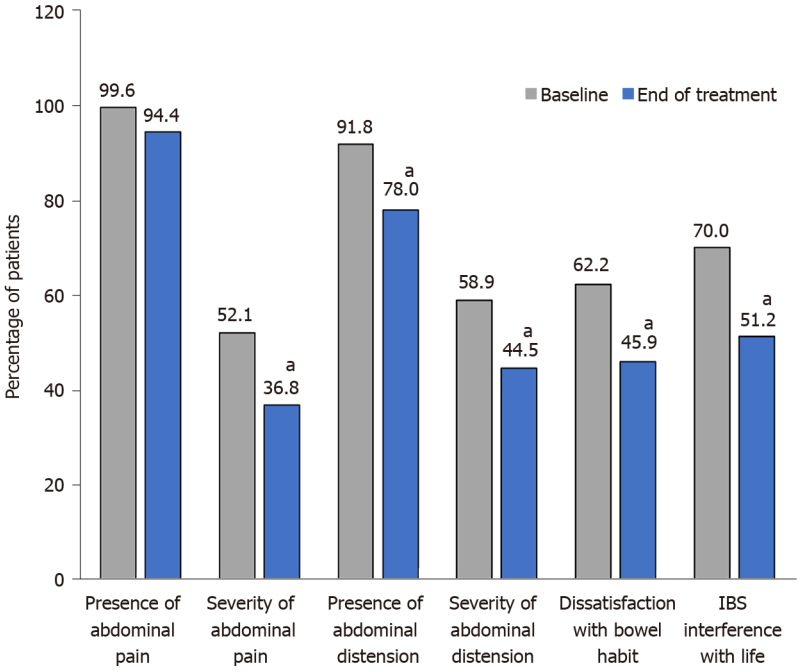


Figure 5 Irritable bowel syndrome severity scoring system scores by domain at baseline and after treatment with *B. longum* 35624. [a]*P* < 0.001 *vs* baseline. IBS-SSS: Irritable bowel syndrome severity scoring system.

magnitude of reduction in disease severity observed in our study is uncommon. Further, during the present study, the majority of patients shifted to lower IBS severity categories, some moving from a severe to a mild form of the disease, which has been associated in the literature with a decrease in health care seeking and an improvement of quality of life[2,21].

Quality of life is often impaired in patients with IBS, especially in those with severe disease. Therefore, the 2014 EMA "Guideline on the evaluation of medicinal products for the treatment of irritable bowel syndrome" recommended the use of validated quality of life scales in order to better understand the real impact of a treatment on the disease[10]. In the present study, overall quality of life measured by the IBS-QOL, a validated questionnaire, was significantly improved, as well as all its individual domains. Comparable levels of improvement in IBS-QOL scores (< 10 points at the group level *vs* placebo) were observed at 3 mo in the trials of drugs such as eluxadoline or linaclotide that have been approved for the treatment of IBS by health authorities in the United States or in Europe[22]. However, in our real-life study a "clinically meaningful" improvement (> 10 points)[23] was observed only in approximately one-third of patients (*i.e.*, half as many as the number of patients who had a significant reduction in IBS severity). The link between disease severity and quality of life observed in

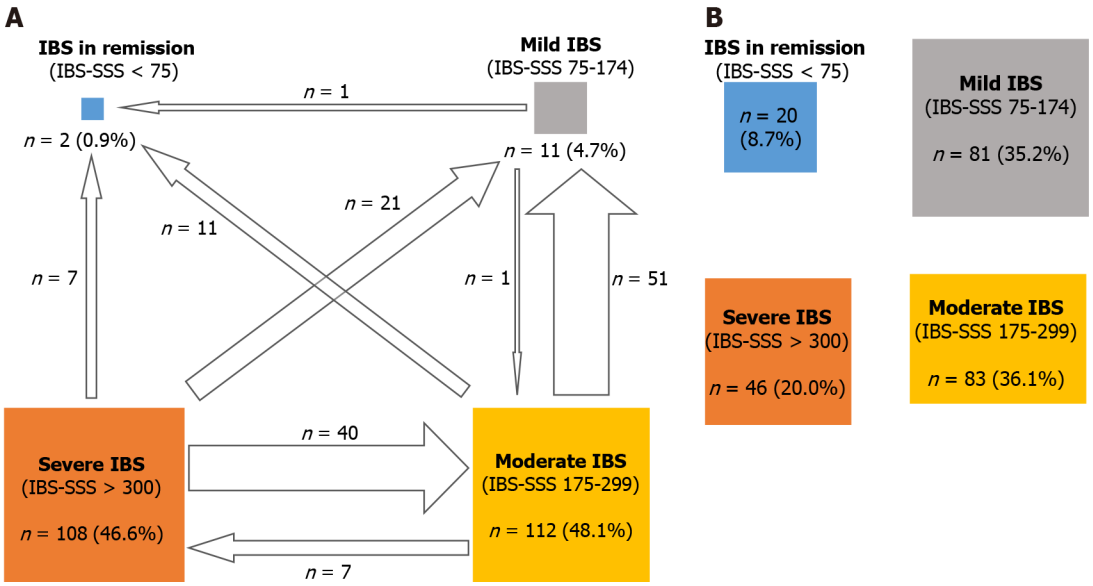


Figure 6 Change in the number of patients in different irritable bowel syndrome severity scoring system severity categories from baseline to after treatment with *B. longum* 35624. A: Number and percentage of patients by Irritable bowel syndrome severity scoring system (IBS-SSS) severity category at baseline (arrows indicate movement from one category to another one after 30 d of treatment with *B. longum* 35624); B: Number and percentage of patients by IBS-SSS severity category after 30 d of treatment with *B. longum* 35624. IBS-SSS: Irritable bowel syndrome severity scoring system.

this study has been previously described in IBS using different quality-of-life instruments[2,24]. The fact that a decrease in severity did not translate into a similar improvement in the quality of life could be because certain behaviors that affect quality of life may require more than 1 mo, which was the duration of the present study, to change (*e.g.*, self-confidence, attitude at work, relationships with others). A similar lag in improvement in the quality of life compared with IBS severity was also observed in a recent study of two other probiotic strains, *Lactobacillus acidophilus* DDS-1 and *B. lactis* UABla-12[11]. Interestingly, a clinically significant improvement in quality of life was observed in our patients with the highest severity of IBS at baseline, suggesting that *B. longum 35624* has also therapeutic potential in patients with severe IBS. The absence of improvement of quality of life in the study conducted by Whorwell *et al*[7] could be explained by the fact that their study probably included fewer patients with severe forms of IBS because it relied on the Rome II criteria for inclusion, and it has been shown that the Rome IV criteria, which are more stringent, increase the percentage of patients with severe forms of IBS [13]. Nevertheless, O'Mahony *et al*[6] have observed a significant improvement in quality of life over placebo in an 8-wk study using the same probiotic strain as in our study[6].

Most of the published studies on probiotics do not target a specific IBS subtype. In our study, the effect of treatment with *B. longum* 35624 over a 30-d period was analyzed according to IBS subtypes. Treatment was effective on each IBS subtype (IBS-C, IBS-D and IBS-M) in terms of disease severity and quality of life. During treatment, we observed the normalization of stool consistency, with a decrease in the frequency of extreme stool types according to the Bristol stool scale (type 1-2 for IBS-C or type 6-7 for IBS-D) and an increase of normal stool type (type 3-5), as it was the case using the same strain in the study of Whorwell *et al*[7].

We found that the incidence of AEs was low (5%), and that AEs were generally minor. This finding is in accordance with the results from a previous large randomized study of *B. longum* 35624[7], confirming its favorable tolerability profile.

This observational study, were only data concerning the patients who took treatment with *B. longum* 35624 are available, had several limitations, namely the absence of a placebo or comparator group and the relatively short treatment duration. Strong placebo effect and a tendency for spontaneous improvement are sometimes described in studies of IBS[25]. For example, in their study, Martoni *et al* [11] observed an average decrease of 30 points in the IBS-SSS score at 3 wk in the placebo group[11]. However, the magnitude of the reduction in severity, which is a relatively stable parameter[2], and the choice of the 50-point reduction in the IBS-SSS score as the threshold[15], which has been previously validated as a reliable indicator of improvement, provide some confidence in the robustness of our results. Even if short, the duration of the present study was sufficient to obtain positive results that are comparable to those of a large randomized study of *B. longum* 35624[7] and three recently published randomized studies of other probiotics[19,26,27]. It should also be noted that, to our knowledge, our study is one of the few[11] that simultaneously assessed severity and quality of life using validated instruments, and one of the few probiotic studies to include patients with IBS diagnosed using the Rome IV criteria. Stool analysis was not performed to correlate improvement of IBS patients' symptoms with a qualitative or quantitative improvement of the intestinal microbiota, but this is rarely done in clinical

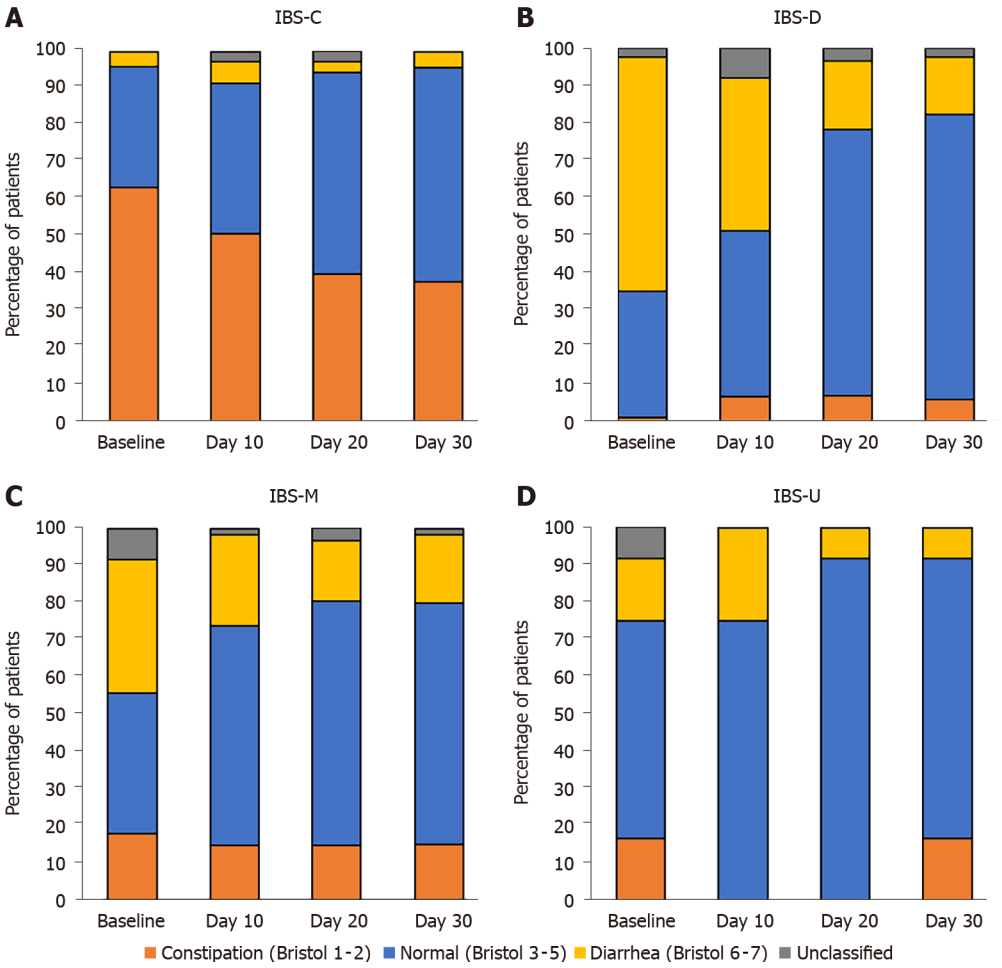


Figure 7 Stool consistency by Bristol stool scale score recorded at baseline and every 10 d over 30 d in IBS-C, IBS-D, IBS-M, and IBS-U.
C: Constipation; D: Diarrhea; IBS: Irritable bowel syndrome; M: Mixed; U: "Unclassified" (subtype not determined). A: IBS-C; B: IBS-D; C: IBS-M; D: IBS-U.

studies. Nevertheless, Charbonneau *et al*[28] using the same probiotic strain observed that after 4 and 8 wk of treatment, fecal levels of *B. infantis* 35624 from IBS subjects who received the probiotic rose significantly compared with those from subjects who received placebo. While in some diseases there may be variations in the microbiota according to ethnicity, this factor could also have influenced the results. However, in France, the legislation does not allow the collection of ethnic data for this type of analysis.

CONCLUSION

This study conducted in IBS patients diagnosed according to the Rome IV criteria and who had different transit pattern subtypes and different levels of symptom severity showed that 30 d of treatment with *B. longum* 35624, whose superiority to placebo has already been established, reduced IBS disease severity and improved patient quality of life in all subgroups of patients, and notably in those with the most severe form of IBS.

ARTICLE HIGHLIGHTS

Research background

Some probiotics have been shown efficacy on irritable bowel syndrome (IBS) symptoms.

Research motivation

However, little data is available on the effectiveness of probiotics on IBS severity and quality of life.

Research objectives
To assess in real life settings efficacy of treatment with *B. longum* 35624 on IBS severity and quality of life.

Research methods
To assess in an observational study on IBS patients defined according to Rome IV criteria, the effect of a 30 d of treatment with *B. longum* 35624 on the disease severity (IBS severity scoring system) and quality of life (IBS quality of life questionnaire).

Research results
After one month of treatment, the severity and quality of life improved in approximately two-thirds and one-third of patients respectively, especially in more severe patients with changes to lower severity categories in more than half of the patients. A gradual improvement in stool consistency was also observed in all transit sub-types.

Research conclusions
In IBS patients defined according to Rome IV criteria, a 30 d treatment with *B. longum* 35624 reduces the disease severity and improves the quality of life even in patients with severe disease that were excluded of most published studies.

Research perspectives
Future research should help to define predictors of good response to probiotic therapy and should study responses to prolonged therapy for this chronic disease.

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FOOTNOTES

Author contributions: Sabaté JM and Iglicki F were responsible for conceptualization, methodology, writing—original draft preparation, and supervision of the study; Sabaté JM conducted the formal analysis and data curation; all authors have read and agreed to the published version of the manuscript.

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Informed consent statement: An information sheet was provided to all patients, and oral non-opposition to the study was obtained from all patients involved in accordance with French regulation.

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Data sharing statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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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ORCID number: Jean-Marc Sabaté 0000-0001-5591-3489; Franck Iglicki 0000-0001-8815-8395.

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SYSTEMATIC REVIEWS

Stereotactic radiotherapy and the potential role of magnetic resonance-guided adaptive techniques for pancreatic cancer

Tai Ermongkonchai, Richard Khor, Vijayaragavan Muralidharan, Niall Tebbutt, Kelvin Lim, Numan Kutaiba, Sweet Ping Ng

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Tai Ermongkonchai, Richard Khor, Sweet Ping Ng, Department of Radiation Oncology, Olivia Newton-John Cancer Centre at Austin Health, Heidelberg 3084, Victoria, Australia

Vijayaragavan Muralidharan, Department of Surgery, Austin Health, Heidelberg 3084, Victoria, Australia

Niall Tebbutt, Department of Medical Oncology, Olivia Newton-John Cancer Centre at Austin Health, Heidelberg 3084, Victoria, Australia

Kelvin Lim, Numan Kutaiba, Department of Diagnostic Radiology, Austin Health, Heidelberg 3084, Victoria, Australia

Corresponding author: Sweet Ping Ng, MBBS, PhD, Associate Professor, Department of Radiation Oncology, Olivia Newton-John Cancer Centre at Austin Health, 145 Studley Road, Heidelberg 3084, Victoria, Australia. sweetping.ng@austin.org.au

Abstract

BACKGROUND

Pancreatic cancer is a malignancy with one of the poorest prognoses amongst all cancers. Patients with unresectable tumours either receive palliative care or undergo various chemoradiotherapy regimens. Conventional techniques are often associated with acute gastrointestinal toxicities, as adjacent critical structures such as the duodenum ultimately limits delivered doses. Stereotactic body radiotherapy (SBRT) is an advanced radiation technique that delivers highly ablative radiation split into several fractions, with a steep dose fall-off outside target volumes.

AIM

To discuss the latest data on SBRT and whether there is a role for magnetic resonance-guided techniques in multimodal management of locally advanced, unresectable pancreatic cancer.

METHODS

We conducted a search on multiple large databases to collate the latest records on radiotherapy techniques used to treat pancreatic cancer. Out of 1229 total records retrieved from our search, 36 studies were included in this review.

RESULTS

Studies indicate that SBRT is associated with improved clinical efficacy and toxicity profiles compared to conventional radiotherapy techniques. Further dose escalation to the tumour with SBRT is limited by the poor soft-tissue visualisation of computed tomography imaging during radiation planning and treatment delivery. Magnetic resonance-guided techniques have been introduced to improve imaging quality, enabling treatment plan adaptation and re-optimisation before delivering each fraction.

CONCLUSION

Therefore, SBRT may lead to improved survival outcomes and safer toxicity profiles compared to conventional techniques, and the addition of magnetic resonance-guided techniques potentially allows dose escalation and conversion of unresectable tumours to operable cases.

Key Words: Magnetic resonance imaging; Pancreatic cancer; Radiotherapy; Stereotactic; Adaptive techniques

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Core Tip: Locally advanced pancreatic cancer has very poor outcomes. These cases are treated with chemoradiotherapy regimens, but conventional radiotherapy techniques often yield minimal survival benefit while accruing significant toxicities. Stereotactic body radiotherapy (SBRT) is an advanced technique that is associated with improved survival outcomes and reduced toxicities compared to its predecessors. The addition of Magnetic resonance-guided techniques to SBRT provides excellent imaging that enables intra-treatment plan adaptations. This provides the possibility of dose escalation, which may be the key to achieving surgical resectability and thus potentially increasing the chances of cure.

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INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer deaths, with a 5-year overall survival (OS) rate of less than 10%[1]. Surgical resection is the only curative option, but is seldom feasible due to a lack of early detection markers, late presentation with locally advanced disease or the lesion being medically inoperable[2-6]. In the cohort of patients who received surgery in the PREOPANC-1 randomised trial, a subgroup analysis in patients with borderline resectable disease demonstrated a survival advantage in those receiving preoperative chemoradiotherapy compared to those receiving immediate surgery[7]. However, the data for definitive radiotherapy (RT) in unresectable pancreatic cancers is conflicting. Traditionally, locally advanced pancreatic cancers (LAPC) unsuitable for curative surgery are treated with chemotherapy regimens or conventionally fractionated radiotherapy (CFRT), or both[8]. However, the role of RT is controversial as radiation-induced toxicities remain a concern. Conventional radiotherapy is often associated with significant grade ≥ 3 toxicities while achieving a median OS of only 5 to 15 mo[2]. The LAP-07 trial demonstrated that the survival outcomes of those who received conventionally fractionated chemoradiotherapy is not superior to chemotherapy alone. However, despite its known caveats, the trial indicated that there is a benefit from RT in multimodal regimens in achieving improved local control (LC), which approached 70% at 12 mo[9].

Stereotactic body radiotherapy (SBRT) is an emerging RT technique due to its ability to deliver highly ablative radiation doses in several fractions[10]. A study by Park *et al*[4] found that the use of a five-fraction SBRT regimen achieved improved quality-of-life scores and tolerable acute toxicities, with comparable late grade ≥ 3 toxicities to intensity-modulated radiotherapy (IMRT) (15.9% SBRT *vs* 13.7% IMRT)[4]. But while SBRT strives for more accuracy and precision, there are some obstacles that prevent further dose escalation without compromising safety. First is the susceptibility of the pancreas to intra-fractional movement during respiratory cycles and digestion. Secondly, the adjacent surrounding organs-at-risk (OAR) which comprises of the stomach, duodenum and small intestine are highly radiosensitive, therefore care needs to be taken to limit doses to these structures to avoid significant treatment-related toxicity. And finally, the current imaging modalities and fiducial markers provide poor visualisation of targets during treatment planning[11].

The recent development of magnetic resonance-guided RT (MRgRT) provides potential to circumvent these challenges, as magnetic-resonance imaging (MRI) offers excellent soft-tissue contrast that can guide dosimetric adjustments to the target volume and limit OAR exposure. This review will evaluate the role of SBRT in the treatment of LAPC, its shortcomings, and present the potential use of MR-guided adaptive techniques to mitigate those caveats.

MATERIALS AND METHODS

Searches were conducted in the online databases PubMed and Ovid (Medline) from August to September 2020, using Medical Subject Headings (MeSH) terms/keywords of pancreatic cancer, stereotactic, radiotherapy and magnetic-resonance. Records were included if it studied the treatment outcomes of SBRT and/or MRgRT in unresectable pancreatic cancers. The excluded literature were review articles or studies done on metastatic disease. Studies that involved resectable tumours or used chemoradiotherapy as adjuvant treatment post-surgery were also omitted. Only results in the English language were included. Additional literature was also sought from references of included studies. A final shortlist of studies was selected based on relevance. A study was considered as relevant if it investigated the effect of SBRT and/or MRgRT on any survival metric in patients with inoperable LAPC.

RESULTS

Figure 1 illustrates the search and screening processes done to assess the eligibility of studies. A total of 1630 records were found from the databases using the search strategy, with an additional 10 retrieved from references of included studies. There was a total of 411 duplicates, and after removal of these we resulted with 1229 records. Screening was conducted by the primary author. The first screening phase was done by screening the titles and abstracts of the 1229 records, which resulted in 93 potential studies. The second screening phase assessed full texts, and 46 further studies were excluded for reasons such as use of novel therapies, investigating metrics not relevant to survival outcomes in pancreatic cancer, or using *in-vivo* animal models. This resulted in 47 eligible texts and out of those, 36 were used to synthesise the discussion. The final 36 texts chosen represented the latest seminal work pertaining to SBRT and MRgRT in treatment of pancreatic cancer.

DISCUSSION

SBRT in patients with LAPC

For patients with unresectable LAPC, chemotherapy has been the mainstay of treatment. Early radiation techniques such as CFRT and IMRT have called into question the value of irradiation in LAPC management due to their considerable toxicity profiles[4,9], with minimal to no impact on survival outcomes[9]. However, SBRT is an advanced radiation technique which can be delivered on the same linear accelerator at most centres. It has gained attraction due to three main reasons: Firstly, it allows delivery of high biologically effective doses (BED) split into several fractions (typically 3-5). Secondly, the technique allows a sharp radiation dose falloff at the edge of target volumes, thereby reducing doses to OARs[12]. Thirdly, it offers an overall shorter treatment time, as SBRT is normally delivered in 1-3 wk, compared to CFRT which takes 5-6 wk[13,14]. Hence, SBRT ensures there is minimal interruption to chemotherapy, which is important given that the main pattern of failure in this disease is distant metastasis (DM)[3]. In addition to this, patients with limited prognoses will be able to complete RT courses in 3 wk instead of 6 wk (which may account for a quarter of their remaining lifespan). This greatly improves quality-of-life, as it requires less commuting and reduces associated costs on patients and families[10,13].

Table 1 summarises the studies of SBRT in unresectable pancreatic cancer. The majority of studies demonstrated an OS of 10-16 mo, freedom from local disease progression (FFLP) rates of approximately 80% and progression-free survival (PFS) of 8-10 mo with SBRT[2-4,13,15-17]. A systematic review by Petrelli *et al*[18] assessed prospective trials and retrospective studies of SBRT use in LAPC, with the pooled results showing a median OS of 17 mo[18]. Other studies compared SBRT's efficacy compared to other RT techniques. A retrospective review by Zhong *et al*[13] showed that patients who received SBRT had improved median OS times and 2-year OS rates relative to CFRT[13]. Similar results were found by Dohopolski *et al*[3] who also demonstrated a higher median OS for the SBRT group (12.6 mo) compared to its counterpart (11.2 mo)[3]. Other studies also showed that patients who had SBRT achieved at least similar outcomes as those who had IMRT[4,19]. Park *et al*[4] demonstrated no significant difference in median OS between those who had SBRT *vs* IMRT[4]. However, Shaib *et al*[19] found that SBRT achieves at least a month longer median OS (8.6 mo *vs* 6.7 mo; $P < 0.001$) and more than double compared to supportive care alone (8.6 mo *vs* 3.4 mo; $P < 0.001$)[19]. However, large prospective trials

Table 1 Studies of stereotactic body radiotherapy in locally advanced pancreatic cancer

Ref.	Participants	Dosimetry	Outcome	Toxicity	Resectability post-treatment
Herman *et al* [15], 2015, Phase 2 Trial	49 LAPC	33 Gy/5 fractions	(1) Median OS 13.9 mo; (2) 59% 1-yr OS; and (3) 18% 2-yr OS	(1) 1 patient acute grade 4 duodenal ulcer; (2) 10% acute grade ≥ 3; (3) 11% late grade ≥ 2; and (4) 6% serious late GI toxicity	10% resectable after treatment
Comito *et al*[2], 2017, Phase 2 trial	43 LAPC	45 Gy/6 fractions	Median OS 13 mo	(1) 49% acute grade 1 or 2; (2) 0 acute grade ≥ 3; (3) 2 patients late G2 gastritis; and (4) 0 late grade ≥ 3	7% resectable after treatment
Dohopolski *et al* [3], 2017, Retrospective	696 LAPC	24-40 Gy/3-5 fractions	Median OS 12.6 mo (compared to 11.2 mo for CFRT)	Not recorded	Not recorded
Park *et al*[4], 2017, Retrospective	44 unresectable	30-33 Gy/5 fractions	(1) 56% 1-yr OS; (2) 26% 2-yr OS; and (3) Median OS 15.7 mo (no significant difference from IMRT)	(1) 7% acute grade ≥ 2 GI toxicity (24% for IMRT); (2) 5% grade ≥ 3 haematological toxicity (26% for IMRT); and (3) 9% late GI bleed	7% resectable after treatment (no significant difference from IMRT)
Yechieli *et al*[10], 2017, Retrospective	18 unresectable	30-36 Gy/3-5 fractions	(1) Median recurrence-free survival 6.8 mo; and (2) Median OS 6.4 mo	(1) 50% no toxicity; (2) 15% grade ≥ 3; and (3) 10% GI bleed	Not recorded
Zhong *et al*[13], 2017, Retrospective	631 LAPC	Median 40 Gy/5 fractions	(1) 22% 2-yr OS (17% for CFRT); and (2) Median OS 13.9 mo (11.6 mo for CFRT)	0 grade ≥ 3	11% resectable after treatment (9% for CFRT)
Mazzola *et al* [14], 2018, Retrospective	33 LAPC	36-45 Gy/6 fractions	(1) 81% 1-yr LC; and (2) 75% 1-yr OS	(1) 15% acute grade 1; (2) 9% acute grade 2; (3) 0 acute grade ≥ 3; and (4) No late toxicity	18% resectable after treatment
Jung *et al*[16], 2019, Retrospective	95 LAPC	24-36 Gy/4-5 fractions	(1) Median OS 16.7 mo; and (2) 67% 1-yr OS	(1) 3% acute grade 3 GI; and (2) 3% late grade 3	7% resectable after treatment
Shaib *et al*[19], 2020, Retrospective	6950 LAPC (64 received SBRT)	Median 30 Gy	(1) Median OS 8.6 mo (6.7 mo for IMRT, 3.4 mo for no RT); (2) 32% 1-yr OS (22% for IMRT, 15% for no RT); and (3) 9% 2-yr OS (7% for IMRT, 5% for no RT)	Not recorded	Not recorded
Toesca *et al*[17], 2020, Retrospective	149 unresectable	20-45 Gy/3-6 fractions (high-dose group ≥ 40 Gy, standard-dose group < 40 Gy)	(1) Median OS 16 mo both groups; (2) Median OS 23 mo for high-dose group (14 mo for standard-dose group); and (3) 82% 1-yr OS for high-dose group (57% for standard-dose group)	(1) 10% grade ≥ 2 for high-dose group (15% for low-dose group); and (2) 6% grade ≥ 3 for high-dose group (7% for low-dose group)	5% resectable after treatment

LAPC: Locally advanced pancreatic cancer; OS: Overall survival; GI: Gastrointestinal; CFRT: Conventionally fractionated radiotherapy; IMRT: Intensity modulated radiotherapy; LC: Local control; SBRT: Stereotactic body radiotherapy; RT: Radiotherapy.

are needed to definitively conclude SBRT's efficacy compared to conventional techniques, but the evidence so far suggests that SBRT is associated with better survival outcomes.

Another advantage of SBRT is its favourable toxicity profiles. Studies in Table 1 report no more than 15% and 10% of patients receiving SBRT suffering from acute grade ≥ 3 toxicities and late side effects (such as duodenal bleeding and gastric ulcer perforation), respectively. Compared to IMRT, SBRT had significantly lower acute grade ≥ 2 gastrointestinal toxicity rates (7% *vs* 24%)[4]. Petrelli *et al*[18]'s systematic review found late grade 3 to 4 toxicity rates of up to 11% in their studies, with only 3 of their included studies reporting > 10% risk of severe gastrointestinal ulceration[18]. The patients of those studies all received higher doses *per* fraction due to previously failed RT[18], suggesting that a relationship exists between delivered doses and toxicity severity in SBRT treatment. The lower toxicity rates may be attributed to SBRT's rapid dose falloffs and the utilisation of motion mitigation methods. The pancreas is a retroperitoneal organ embedded around gastrointestinal structures, hence it undergoes significant motion during respiratory cycles and physiological processes such as digestion. The two commonly used motion mitigation methods during SBRT are respiratory gating and abdominal compression. Respiratory gating uses an external surrogate marker that represents the internal tumour position, where the radiation beam is only delivered when this marker correlates to a certain phase of the respiratory cycle. Abdominal compression requires applying pressure onto the abdomen to suppress diaphragmatic movements, but is less preferred due to patient discomfort and the occasional

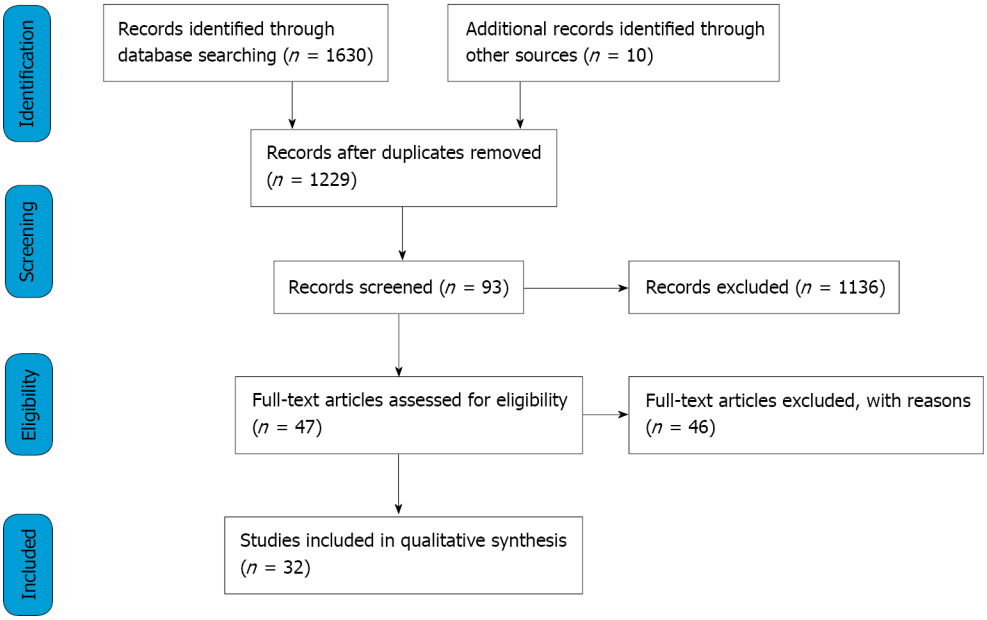


Figure 1 PRISMA diagram of literature search.

displacement of OARs closer to the radiation volume[11]. A prospective study by Campbell *et al*[11] confirmed that both methods reduce motion and OAR exposure compared to no mitigation, however respiratory gating achieves greater motion reduction than abdominal compression by more than 20% [11].

Interestingly, while the studies only included unresectable patients, a small proportion were able to receive surgical resection after their SBRT course. As shown in Table 1, the rate of conversion to surgical resectability by SBRT was 5%-18%. In these studies, surgical resectability was decided upon multidisciplinary review including operating surgeons. This is important because if SBRT can induce local tumour regression and subsequently convert the tumour from unresectable to resectable, then it can possibly improve the chances of cure. The study by Mazzola *et al*[14] yielded the highest rates of resectability at 18%, all of which were participants that received higher doses of SBRT at 42-45 Gy in 6 fractions[14]. Meanwhile, Petrelli *et al*[18] found that higher total doses and number of fractions are significantly associated with 1-year locoregional control[18]. These results suggest that dose escalation may be the key determinant in achieving LC and thus conversion to surgical resectability. Currently, for five-fraction regimens, dose escalations of up to 60 Gy is feasible without compromising adequate target coverage and OAR constraints[20,21].

Alternative fractionation schemes

Recent evidence indicates that patients may benefit from alternative fractionation regimens, especially for those with gross tumour abutment into surrounding structures or invasion into peripancreatic nodes. The rationale is to prolong the treatment regime (≥ 10 fractions) such that higher overall BEDs can be delivered while still accounting for OAR toxicity. Reyngold *et al*[22] studied ablative schemes of 75 Gy in 25 fractions (BED = 97.5 Gy) and 67.5 Gy in 15 fractions (BED = 97.88 Gy) for patients with significant tumour abutment to the stomach/intestines, demonstrating a median OS of 18.2 mo and a 2-year OS of 38%[22]. This is an improvement from standard 1-5 fraction regimens, as the reported 2-year OS from those studies ranged from 9%–26%[4,13,15,19].

Caveats of current SBRT

Despite the advances of SBRT, its overall management of LAPC is limited by its imaging modalities. SBRT utilises computed tomography (CT)-based techniques such as 4-Dimensional CT (4DCT) and Cone Beam CT to assess tumour movement and carry-out the motion mitigation techniques[23]. This is a limitation because CT has poor soft-tissue contrast and is unable to accurately determine the appropriate therapy volumes. Furthermore, CT often involves larger planning target volumes (PTV) or use of an internal target volume (ITV) to account for tumour motion, thus putting the surrounding OARs at increased toxicity risk and ultimately preventing any possibility of dose escalation[24]. Furthermore, 4DCT only provides the average of motion amplitude over several respiratory cycles. Since the fourth dimension represents "phase" of respiration rather than being real-time, tumour motion might even be underestimated[25]. This explains why despite SBRT's evidence in reducing acute toxicity, there are still significant concerns with late toxicity as previous published studies report rates of up to 47% of late grade ≥ 2 toxicity[2]. Therefore, SBRT is constrained by dose-limitations placed on the

surrounding OARs. Another concern is its steep dose gradient and the marginal misses that may result [26]. This is made more challenging given that conventional CT tends to underestimate the true pathologic size of the pancreatic tumour[27]. To optimise SBRT's therapy volumes and dose distribution, a better imaging modality needs to be incorporated.

Emerging role of MRgRT

MRgRT has been proposed as the solution to the inconsistencies of onboard imaging with RT. MRI provides superior soft-tissue visualisation compared to CT and thus allows better delineation of the target tumour from surrounding OARs. Its real-time feedback also tracks inter-fractional and intra-fractional organ changes[8,28,29]. Another benefit of MRI is its exploration of multiple breathing cycles over different days to quantify daily changes[25]. Therefore, MRgRT can be used to guide treatment plan adaptations, such that therapy volumes account for intra-treatment tissue changes[30]. This led to the advent of Stereotactic MR-guided Adaptive Radiotherapy (SMART), which is the application of the principles of MRgRT combined with SBRT. A non-randomised trial by Heerkens *et al*[25] assessing the feasibility of MRgRT with SBRT showed that it is safe with dosimetric plans of at least 24 Gy, with no cases of acute or late grade ≥ 3 toxicity. They were also able to deliver higher doses under free-breathing conditions while ensuring adequate target coverage and OAR sparing[25]. SMART has become a promising technique in LAPC by possibly enabling SBRT dose escalation without exposing OARs to higher toxicity risk[8,25].

Table 2 summarises recent studies of MRgRT use in LAPC. Rudra *et al*[31] investigated the use of MRgRT with standard-dose and high-dose SBRT plans, and were able to demonstrate that dose escalation is possible. Patients in the high-dose group (receiving 40-52 Gy) achieved significantly higher survival rates compared to those in the standard-dose group (receiving 30-35 Gy), despite the former cohort having worse prognostic factors such as older age and higher Carbohydrate Antigen 19-9 biomarker levels[31]. There was no incidence of severe toxicity amongst the higher dose group, with all cases of grade ≥ 3 gastrointestinal toxicities reported from the standard-dose cohort[31]. A study by Luterstein *et al*[8] on a patient case yielded similar results. The patient with clinical stage III (T4N1M0) LAPC was given a high BED of 72 Gy *via* SMART after chemotherapy and achieved LC at 16 mo post-radiation (21 mo since diagnosis) with no significant side effects or toxicities[8]. Furthermore, a multi-institutional study at the American Society for Radiation Oncology suggested that adaptive plans that allow safe delivery of BED > 70 Gy can achieve higher OS rates than BED < 70 Gy without impacting surrounding OARs[8]. These results indicate that MRgRT's precision can potentially address prior issues with RT. And since previous studies recommend that five-fraction regimens should use a dose prescription of 40 Gy to cover the gross tumour[32], the advances of MRgRT provides potential to maximise this limit in the future without compromising safety.

With the implementation of MRgRT in its early stages, some caveats have emerged such as workflow disruptions. Utilising MRI to guide therapy also poses new challenges unique to the MRI magnet, including but not limited to patient selection and MRI safety. This needs particular consideration as the MRI magnet is now being used outside of a radiology department where MRI safety protocols are firmly embedded into work practices. As with any novel modality or technological advancement, there will be a learning curve and an initial period to bolster awareness of safety requirements.

Concomitantly, adaptive techniques also require increased time investment as plans need to be re-optimised between fractions. Hence, MRgRT is costly and resource-intensive because it involves multidisciplinary teams to re-contour images, review and re-approve the adapted plans daily[28,30]. There is now an emerging interest to use artificial intelligence tools such as auto-contouring methods and radiomics to increase the workflow efficiency of treatment planning.

Strengths and limitations of the review

Our review methodology covers a wide range of literature, but it comes with limitations. The review mostly sought evidence from large retrospective studies without individual data for each patient. Hence, it was difficult to identify confounding factors that may exist due to the variability of patient characteristics. The review also excluded studies on patients with DM as it aimed to investigate SBRT's effect locally. This may artificially elevate survival rates as those without DM will naturally have better outcomes.

The included evidence came with strengths and limitations. Firstly, SBRT has mature follow up data from several large retrospective analyses, with evidence dating back over a decade. This provided ample evidence to suggest that SBRT is a safe and beneficial technique for multimodal management of LAPC. However, the heterogeneity in study designs contributes to a large variability in the data. Since LAPC management differs on a case-by-case basis according to tumour staging and the physician's clinical judgement, many of these studies include patient cohorts that received different chemotherapy regimens from each other. As a result, it is unsure how much survival benefit can be attributed to SBRT. It is also noteworthy that many of these studies could involve selection bias, since the most unwell patients often received no treatment and went into palliative care. This led to "healthier" subjects chosen for SBRT and thus better OS rates. Many of the included studies are retrospective analyses of database records, presenting another source of selection bias. Meanwhile, there is limited research on MRgRT so far, thus definitive conclusions about this technique cannot be made. There is a need for large

Table 2 Studies of magnetic resonance-guided radiotherapy in locally advanced pancreatic cancer

Ref.	Participants	Dosimetry	Outcome	Toxicity
Heerkens *et al*[25], 2018, Trial	20 (18 LAPC, 2 unresectable)	24 Gy/3 fractions	(1) Median OS 8.5 mo; (2) 69% improved QOL compared to baseline at 1 mo; and (3) 33% improved QOL compared to baseline at 12 mo	No grade ≥ 3 acute or late toxicity
Luterstein *et al*[8], 2018, Case Report	1 LAPC	40 Gy/5 fractions	LC at 16 mo	None
Rudra *et al*[31], 2019, Retrospective	44 unresectable (22 received SBRT)	30-35 Gy/5 fractions (standard-dose group, n = 6); 40-52 Gy/5 fractions (high-dose group, n = 16)	(1) 49% 2-yr OS (high-dose group); (2) 30% 2-yr OS (standard-dose group); (3) 77% 2-yr FFDF (high-dose group); and (4) 57% 2-yr FFDF (standard-dose group)	Acute: (1) 7% grade ≥ 3 GI (all in standard-dose group); and (2) 2% grade 4

LAPC: Locally advanced pancreatic Cancer; OS: Overall survival; QOL: Quality of life; LC: Local control; SBRT: Stereotactic body radiotherapy; FFDF: Freedom from distant failure; GI: Gastrointestinal.

prospective trials on SBRT and MRgRT, with comparisons to other treatment modalities to validate the results of previous retrospective studies. However, given LAPC's generally poor outcomes, long-term prospective studies will be challenging.

CONCLUSION

SBRT is an advanced radiation technique that allows delivery of ablative doses in several fractions. It is highly precise, time-efficient and can limit OAR exposure when combined with motion mitigation techniques. SBRT is associated with improved treatment outcomes and safer toxicity profiles compared to other conventional RT techniques. And by implementing MR-guided imaging techniques with SBRT, the excellent soft-tissue contrast of MRI enables the physician to make daily plan adaptations such that target volumes are optimised according to intra- and inter-fractional tissue changes. This enables the possibility of dose escalation, which may be the key in achieving long-term LC and converting unresectable LAPC into operable cases. The current evidence on MR-guided SBRT is still limited, but early protocols have suggested its promise. Further research should focus on validating the feasibility, safety and efficacy of MRgRT with comparison to other treatment modalities.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is associated with significant mortality, and unresectable tumours are commonly treated with chemoradiotherapy regimens. Conventional radiotherapy (RT) techniques have minimal impact on survival and often cause considerable toxicities. Stereotactic body radiotherapy (SBRT) is an advanced radiotherapy technique that delivers highly ablative doses in several fractions, with a steep dose fall-off outside target volumes.

Research motivation

Previous studies have supported the benefit of radiotherapy in multi-modal management of unresectable pancreatic cancers. However, there is no consensus of which RT technique yields the best survival outcomes. There is also a need for research to explore onboard imaging such as magnetic resonance-guided radiotherapy (MRgRT), which will enable treatment plans to be optimised according to intra-treatment tissue changes.

Research objectives

We aim to collate the latest data on SBRT and evaluate its survival outcomes and toxicity profiles, with comparison to conventional RT techniques. Our review will also cover the safety and efficacy of MRgRT.

Research methods

Searches were conducted on PubMed and Ovid (Medline), resulting in 1229 records. After multiple rounds of screening, 36 texts were chosen to synthesise the discussion. Records were included if they studied SBRT or MRgRT in unresectable cancers, and excluded if they involved metastatic disease, resectable tumours or used chemoradiotherapy as adjuvant to surgery.

Research results

SBRT is associated with improved survival outcomes and toxicity profiles compared to conventional RT techniques. A small proportion of unresectable patients were able to undergo surgical resection after their SBRT course. Conversion to resectability was associated with higher doses. However, dose escalation in SBRT is limited by the onboard computed tomography (CT) imaging due to its poor soft-tissue contrast. MRgRT may address these issues as magnetic resonance imaging (MRI) provides excellent tissue visualisation and is appropriate for real-time scanning. Early data indicates MRgRT as a safe and efficacious technique.

Research conclusions

SBRT may lead to improved survival outcomes and safer toxicity profiles compared to conventional RT, but is ultimately limited by onboard CT imaging. The addition of MRI-guided techniques allows the potential for dose escalation, which may be the key to achieving surgical resectability and possibly increasing the chances of cure.

Research perspectives

There is a need for large prospective trials to definitively conclude if SBRT is superior to other RT techniques. Large studies are also required to validate the safety, feasibility and efficacy of MRgRT with comparison to other RT techniques.

FOOTNOTES

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Country/Territory of origin: Australia

ORCID number: Tai Ermongkonchai 0000-0002-2210-5773; Richard Khor 0000-0002-7057-2747; Vijayaragavan Muralidharan 0000-0001-8247-8937; Niall Tebbutt 0000-0003-2613-5168; Kelvin Lim 0000-0002-5216-8994; Numan Kutaiba 0000-0003-4627-9847; Sweet Ping Ng 0000-0003-1721-0680.

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CASE REPORT

Crohn's disease-related 'gastrocnemius myalgia syndrome' successfully treated with infliximab: A case report

Julien Catherine, Hazim Kadhim, Frédéric Lambot, Claire Liefferinckx, Virginie Meurant, Lukas Otero Sanchez

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Julien Catherine, Institute for Medical Immunology, Université Libre de Bruxelles, Gosselies 6041, Belgium

Julien Catherine, Department of Internal Medicine, C.U.B. Hôpital Erasme, Brussels 1070, Belgium

Hazim Kadhim, NeuroMyopathology Unit (Anatomic Pathology Service) and Reference Center for Neuromuscular Pathology, CHU Brugmann, Université Libre de Bruxelles, Brussels 1020, Belgium

Frédéric Lambot, Department of Internal Medicine, Centre Hospitalier Universitaire Tivoli, La Louvière 7100, Belgium

Claire Liefferinckx, Lukas Otero Sanchez, Department of Gastroenterology, Hepatopancreatology and Digestive Oncology, C.U.B. Hôpital Erasme, Brussels 1070, Belgium

Claire Liefferinckx, Lukas Otero Sanchez, Laboratory of Experimental Gastroenterology, Université Libre de Bruxelles, Brussels 1070, Belgium

Virginie Meurant, Department of Emergency Medicine, Centre Hospitalier Universitaire Tivoli, La Louvière 7100, Belgium

Corresponding author: Julien Catherine, MD, Medical Assistant, Research Fellow, Institute for Medical Immunology, Université Libre de Bruxelles, Rue Adrienne Boland 8, Gosselies 6041, Belgium. julien.catherine@ulb.be

Abstract

BACKGROUND

Extra-intestinal manifestations in inflammatory bowel diseases (IBD) are frequent and involve virtually all organs. Conversely, the clinical characteristics and course of inflammatory myopathies in IBD remain poorly described and mostly related to orbital myositis. Moreover, alternative therapeutic strategies in non-responder patients to corticosteroid therapy must still be clarified.

CASE SUMMARY

A 33-year-old woman with a history of unclassified colitis presented with acute bilateral calf pain. On admission, her clinical and biological examinations were non-specific. However, magnetic resonance imaging showed bilateral inflammatory changes in gastrocnemius muscles suggestive of myositis. Muscle biopsy confirmed the diagnosis of myositis and demonstrated an inflammatory infiltrate

mainly located in the perimysial compartment including lympho-plasmocytic cells with the formation of several granulomatous structures while the endomysium was relatively spared. The combined clinical, biological and histomyopathological findings were concordant with the diagnosis of 'gastrocnemius myalgia syndrome' (GMS), a rare disorder associated with Crohn's disease (CD). Ileocolonoscopy confirmed CD diagnosis and systemic corticosteroids (CS) therapy was started, resulting in a rapid clinical improvement. During CS tapering, however, she experienced a relapse of GMS together with a severe active ileocolitis. Infliximab was started and allowed a sustained remission of both conditions at the latest follow-up (20 mo).

CONCLUSION

The GMS represent a rare CD-associated inflammatory myopathy for which anti-tumour necrosis factor-α therapy might be considered as an effective therapeutic option.

Key Words: Crohn's disease; Extra-intestinal manifestation; Gastrocnemius myalgia syndrome; Granulomatous myositis; Anti-tumour necrosis factor-α therapy; Case report

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Core Tip: Inflammatory myopathies are scarce in the setting of inflammatory bowel diseases (IBD) and could be wrongly attributed to IBD-related osteoarticular manifestations or to medications' side effects. This case describes a very atypical presentation of myositis restricted to the legs called the 'gastrocnemius myalgia syndrome', an entity only described during Crohn's disease. Its restricted location to the legs as well as normal creatine kinase levels in serum reflecting a predominant interstitial immune reaction are key characteristics in reported cases. Although corticosteroids are often used as first-line therapy, cortico-dependence is not rare and anti-tumour necrosis factor-α agents might represent an effective therapeutic option.

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INTRODUCTION

The multi-system nature of inflammatory bowel diseases (IBD) is widely known. The extraintestinal manifestations (EIMs) of IBD are diverse and deferred from IBD diagnosis in a quarter of the cases. It has been estimated that 6% to 47% of patients with IBD experience at least one EIM[1]. The most frequently reported EIMs include peripheral and/or axial arthropathy, cutaneous lesions, ophthalmological immune-inflammatory manifestations and hepatobiliary affections[2]. However, EIMs can involve almost any organ or tissue, and some rare manifestations, such as specific muscular involvement can also occur and might even herald an hidden IBD[3].

The occurrence of muscular involvement in IBD is scarce, and mostly related to orbital myositis, a subtype of orbital pseudotumor principally reported during Crohn's disease (CD)[4,5]. Myositis affecting the limbs is a much rarer entity, and is mostly localized in the lower extremities, involving the gastrocnemius muscles, a clinical entity designated as "gastrocnemius myalgia syndrome" (GMS)[3]. To date, very little is known about the full spectrum of myositis occurring during the course of IBD.

We hereby report the first CD-associated GMS in a Belgian patient that turned out to be "granulomatous myositis", an exceptionally rare subtype. In addition, we performed a literature review by searching EMBASE, MEDLINE, Scopus, and the Cochrane Library databases for studies published between January 1, 1970, and July 7, 2021, using the following keywords or MeSH terms: 'Inflammatory Bowel Diseases', 'Crohn's Disease', 'Ulcerative colitis', 'Myositis', 'Gastrocnemius myalgia syndrome' and 'Granulomatous Myositis'. Finally, we discuss the role of anti-tumour necrosis factor (TNF)-α therapy in this specific context.

CASE PRESENTATION

Chief complaints

A 33-year-old woman presented at the emergency department with a 5-d history of isolated tenderness in both calves leading to walking difficulties.

History of present illness

She denied any traumatism but reported a colitis relapse 30 d before her admission which resolved with a short-course of modified-release beclomethasone therapy.

History of past illness

The patient had a history of allergic asthma, *Heliobacter pylori* gastritis and unclassified colitis. Previous investigations performed during colitis flares did not discriminate a specific IBD pattern concluding in an unclassified colitis. The patient was not taking any chronic medication. Her familial history was not contributive.

Physical examination

Clinical findings on admission included bilateral swelling of both calves which were warm and painful to palpation. Motricity and sensitive perception were preserved. Examination of other muscles, joints and the spine was unremarkable and her abdomen was soft and non-tender.

Laboratory examinations

Initial laboratory investigations showed a C-reactive protein level at 106.6 mg/L (normal range, 0.4-12 mg/L) with mild neutrophilic leukocytosis (8770/mm^3, normal range 1900-8000/mm^3). Creatinine kinase (CK), aspartate aminotransferase, lactate dehydrogenase and D-dimer serum levels were within reference values. Hemocultures were negative as well as antinuclear, antineutrophil cytoplasmic and anti-*saccharomyces cerevisiae* antibodies.

Imaging examinations

Ultrasonography of lower limbs revealed a bilateral 5-millimeter-thick edema surrounding gastrocnemius muscles in the absence of vascular abnormality while a magnetic resonance imaging showed marked inflammation of muscles and their fascia in both legs, suggested by a high signal on T2-weighted images and a strong gadolinium enhancement on fat-suppressed T1-weighted images (Figure 1).

Muscular biopsy

A muscular biopsy of the right gastrocnemius revealed a remarkable inflammatory reaction that particularly involved the perimysial compartment. The infiltrate mainly comprised dense lymphoplasmocytic cells with the formation of several granulomatous structures wherein several histiocytes, sometimes multinucleated, were observed (Figure 2A, C and D). Endomysial inflammatory infiltrate was however less remarkable and rather discrete and focal. There was besides a very mild and discrete perivascular affinity for the inflammatory infiltrate but there was no frank/convincing vasculitis in the examined sections (Figure 2B). A few muscle fibers showed suspected myofibrillary disintegration. Immunohistochemical analyses showed an outstanding cohort of CD3 positive cells (with a slight predominance of CD8$^+$ over CD4$^+$ cells) (Figure 2C and D). Electron microscopy was generally unremarkable and showed rare lipofuscin deposits. Moreover, rare mastocytes were observed.

FINAL DIAGNOSIS

This histomyopathological affection in this clinical context was concordant with the GMS, a rare disorder classically associated with CD[6]. Moreover, a new ileocolonoscopy was performed during the hospital stay and revealed endoscopic pattern in favor of CD diagnosis, despite the absence of intestinal granuloma on histopathologic examination.

TREATMENT

Considering these results, the patient was started on a prednisone equivalent dose of 1 mg/kg, resulting in a rapid clinical and biochemical remission.

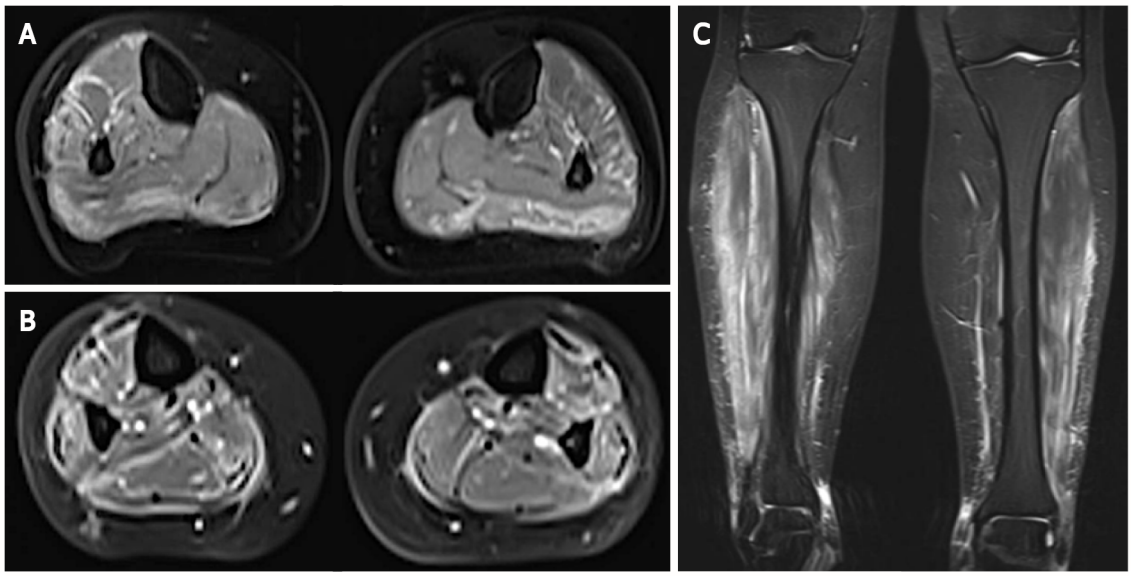


Figure 1 Magnetic resonance imaging of the calves in axial and frontal views. A and C: Demonstration of high signal on T2-weighted images in muscles from posterior and lateral compartments of the legs as well as in their surrounding fascia; B: This finding was accentuated on gadolinium-enhanced fat-suppressed T1-weighted images, suggestive of myositis and fasciitis.

OUTCOME AND FOLLOW-UP

Two months later, the patient experienced intestinal symptoms associated with a recurrence of pain in both legs during corticosteroids (CS) tapering. The ileocolonoscopy performed at that time showed a very severe ileocolitis [CD Endoscopic Index of Severity (CDEIS) scored at 31] with no histopathologic evidence of granuloma for which intravenous methylprednisolone (1 mg/kg for 5 d) and infliximab (5 mg/kg at week 0, 1 and 4) therapies were started to target both muscular and intestinal disease leading to clinical remission of both ileocolitis- and myositis-related symptoms. Oral corticotherapy was then prescribed and slowly tapered while infliximab was continued (5 mg/kg, every 8 wk). Twenty months later, both ileocolitis and myositis were quiescent.

DISCUSSION

Inflammatory myopathies (IM) occurring in the setting of IBD are considered as rare EIMs and have been roughly described in the literature to date[6]. We report the first CD-associated GMS in a Belgian patient that besides turned out to be "granulomatous". This represents a very exceptional subtype as there has been only two such reported cases. To the best of our knowledge, only 15 cases of GMS are described in the international literature and always occurred in patients with CD (Table 1). Apart from GMS, orbital myositis, which is currently considered as a subtype of idiopathic orbital inflammation, has also been reported in patients with IBD, mainly CD[5]. Few cases of dermatomyositis (DM) and polymyositis have also been described during the course of IBDs and a recent study showed that DM was more frequent in ulcerative colitis patients than CD patients and control subjects[7]. Nevertheless, the true incidence of IM occurring in this context is probably underestimated as myalgia can be confused with joint manifestations or with non-inflammatory muscle disorders (*e.g.*, secondary to hypokalemia or medications) and by the fact that clinicians may rule out myositis' diagnosis in the absence of CK elevation in serum.

The first case of myositis affecting gastrocnemius muscles in CD was reported in 1979 by Ménard *et al* [8] who described a 44-year-old man with granulomatous myositis localized to the calf occurring two months before CD diagnosis[8]. Since then, several cases have been reported, sharing the classically following features: (1) Calf-limited myalgia revealing localized myositis; (2) Normal serum CK levels; and (3) A high early-response rate to CS therapy[9]. In 2003, this entity was denominated as "GMS" by Christopoulos *et al*[3], a term adopted in the literature ever since[10]. Most patients developed GMS months or years after the onset of CD but myositis could precede gastrointestinal manifestations by up to 10 years[11]. When CD had been diagnosed before GMS, the intestinal disease was active in most cases at myositis' diagnosis (Table 1). Other EIMs were associated with GMS in 50% of patients which is in accordance with previous observations that patients who presented an EIM are at higher risk to develop another one[1].

Table 1 Case descriptions of gastrocnemius myalgia syndrome reported in Crohn's disease

Ref.	Age (yr)/sex at GMS onset	Initial presentation, time-to-onset	Active CD at time of GMS dx	CK serum level	Biopsy	Treatment	Corticosteroids-dependence (d) or resistance (r)
Ménard *et al*[8], 1976	44/M	Muscular, 2 mo	No	N	Granulomatous myositis	PDS[1] (80 mg/d)	No
Gilliam *et al*[13], 1981	19/M	Digestive, 6 mo	Yes	N	Necrotizing vasculitis	PDS[1] (60 mg/d)	No
Hall *et al*[10], 1985	32/F	Muscular, 120 mo	Yes	N	Non-granulomatous myositis	5-ASA[1], PDS[1] (25 mg/d)	No
Drabble and Gani [14], 1992	50/M	Digestive, 168 mo	Yes	N	Not performed	Hydrocortisone[1] (400 mg/d), PDS[1]	No
Disdier *et al*[11], 1997, Case 1	26/F	Muscular, 48 mo	Yes	N	Necrotizing vasculitis	PDS[1] (60 mg/d), CYC[1], 5-ASA[1]	Yes (r)
Disdier *et al*[11], 1997, Case 2	21/F	Simultaneous	Yes	N	Vasculitis	PDS (1 mg/kg/d), AZA[1]	Yes (d)
Christopoulos *et al*[3], 2003	19/F	Simultaneous	Yes	N	Granulomatous myositis	PDS[1] (0.5 mg/kg/d)	No
Ullrich *et al*[15], 2009	25/F	Digestive, 84 mo	Yes	N	Vasculitis	PDS (50 mg/d), AZA, IFX[1]	Yes (d)
Co *et al*[16], 2010	15/F	Digestive, 8 mo	Yes	N	Not performed	IFX[1]	/
Mogul *et al*[17], 2010	15/M	Digestive, 60 mo	No	N	Non-granulomatous myositis	PDS[1] (40 mg/d), MTX[1]	No
Piette *et al*[18], 2010	45/M	Simultaneous	Yes	(3-13 × N)	Vasculitis	PDS[1] (1 mg/kg/d)	No
Goldshmid *et al* [19], 2011	24/F	Simultaneous	Yes	(330 U/L)	Not performed	MPDS (100 mg/d), IFX[1], AZA[1]	Yes (d)
Vadala di Prampero *et al* [20], 2016	26/M	Digestive, 72 mo	Yes	N	Non-granulomatous myositis	PDS (60 mg/d), AZA[1] (chronic), Adalimumab[1]	Yes (d)
Saffar[12], 2017	33/F	Digestive, 120 mo	Yes	N	Not performed	PDS, IFX[1]	Yes (r)
Osada *et al*[9], 2018	38/M	Digestive, 3 mo	Yes	N	Non-granulomatous myositis	PDS, AZA[1], 5-ASA[1]	Yes (d)
Current case	33/F	Simultaneous	Yes	N	Granulomatous myositis	MDPS (0.8/kg/d), IFX[1]	Yes (d)

[1]These treatment modalities are those that induced a persistent remission of myositis-related symptoms.

GMS: Gastrocnemius myalgia syndrome; CD: Crohn's disease; MDPS: Mitochondrial-derived peptides; IFX: Infliximab; ASA: Aminosalicylate; AZA: Azathioprine; PDS: Peroxydisulfate.

While clinical and biological features in patients with GMS are generally characteristics, histomyopathological findings are rather heterogeneous. In fact, our case is only the third in which granulomatous lesions were observed while all remaining reported cases were characterized by non-granulomatous inflammation (4 cases) or localized vasculitis (5 cases) (Table 1). However, whatever the histopathologic image observed, the inflammatory infiltrate was more often localized in the perimysium and more discrete in the endomysium, probably explaining why most patients with GMS have normal CK values. GMS could therefore represent a form of localized "perimyositis" with no or limited myofiber injury. This observation also suggests that the immune response is directed against connective tissue components or vessels rather than against the myofibers themselves.

Finally, while GMS was initially controlled in this case by oral CS therapy, it ultimately relapsed during tapering. In this context, the presence of a concomitant severe active ileocolitis prompted us to start an anti-TNF-α agent. While CS were the first-line treatment prescribed in almost all cases of GMS, their efficacy, however, was not enduring as 6/16 patients (including ours) relapsed during dose de-escalation (Table 1). Moreover, two other patients were initially refractory to CS[12,13]. Various drugs were used in these corticosteroid-dependent or refractory cases. Anti-TNF-α agents were constantly associated with GMS remission in the six cases where they were introduced, suggesting that the

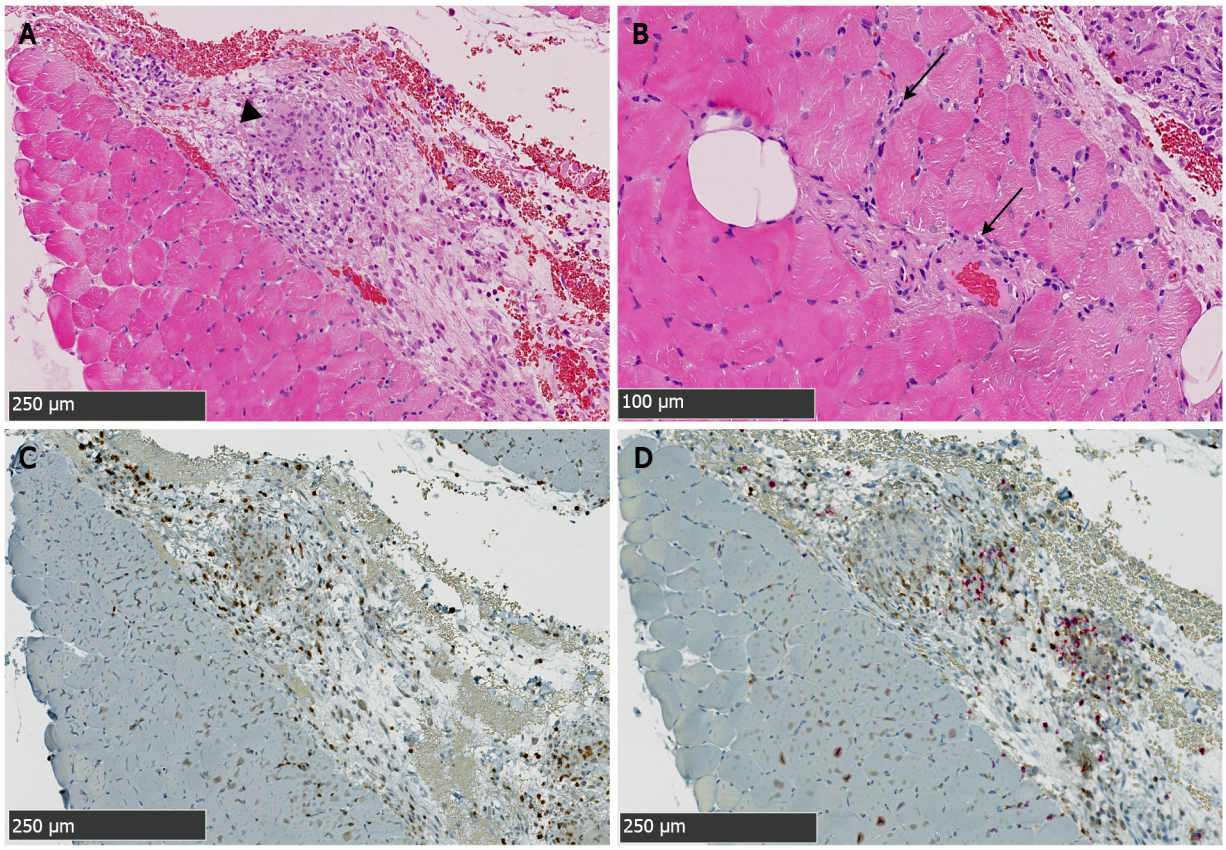


Figure 2 Biopsy specimen from the right gastrocnemius muscle showing the intense immune-inflammatory reaction. A: This hematoxylin and eosin-stained section shows the granulomatous nature (arrowhead) of this Crohn's disease-associated skeletal myositis, predominating in the perimysial compartment; B: Discrete and focal immune infiltrates are found in the endomysium and around vascular structures (arrows); C and D: Immunohistochemical staining characterize immune cell-types involved therein: Co-staining for CD3 (brown staining) and CD20 lymphocytes (red staining) shown in C reveals predominance of $CD3^+$ lymphocytes over $CD20^+$ cells. CD4 and CD8 T cells subtyping shown in D (brown and red staining respectively) further reveals a slight predominance of $CD8^+$ over $CD4^+$ cells.

muscular and intestinal affections in this context could share a common pathophysiological mechanism. Importantly, our case represents the first GMS with granulomatous inflammation treated with an anti-TNF-α agent. Further case descriptions and/or case series will be required to ascertain the role of such therapy in this specific context.

CONCLUSION

In conclusion, we report a rare extra-intestinal manifestation of CD, namely a GMS, characterized by a very rarely reported granulomatous perimyositis. The rapid relapse of GMS during CS tapering and the emergence of a severe active ileocolitis prompted the introduction of an anti-TNF-α agent that resulted in persistent clinical remission of both disorders. This might further suggest the effectiveness of such agents in CS-dependent or refractory cases. Finally, physicians should remain aware of the eventual emergence of a such complicating myositis when musculo-skeletal symptoms arise in IBD patients even in the absence of CK elevation.

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FOOTNOTES

Author contributions: Lambot F, Catherine J and Otero Sanchez L managed the patient; Kadhim H did myopathology

diagnosis and analyses; Catherine J, Otero Sanchez L and Meurant V designed the study and collected data; Catherine J and Otero Sanchez L drafted and wrote the manuscript; Liefferinckx C, Lambot F and Kadhim H provided their expertise throughout the course of the work by revising and editing the manuscript; all authors approved the final version of the manuscript to be published.

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Informed consent statement: The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the institutional review board of CHU Tivoli (approval number #1396). Patient's informed consent was obtained by Meurant V.

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Country/Territory of origin: Belgium

ORCID number: Julien Catherine 0000-0001-8825-8126; Hazim Kadhim 0000-0003-4943-9172; Frédéric Lambot 0000-0003-3585-3444; Claire Liefferinckx 0000-0002-2046-4051; Virginie Meurant 0000-0002-7626-8543; Lukas Otero Sanchez 0000-0002-8013-1371.

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LETTER TO THE EDITOR

Gallbladder biliary lithotripsy: A new rationale applied to old treatment

Lorenzo Dioscoridi, Massimiliano Mutignani

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Lorenzo Dioscoridi, Massimiliano Mutignani, Digestive Endoscopy Unit, ASST Niguarda, Milan 20100, Italy

Corresponding author: Lorenzo Dioscoridi, MD, PhD, Surgeon, Teacher, Digestive Endoscopy Unit, ASST Niguarda, Piazza dell'Ospedale Maggiore 3, Milan 20100, Italy.
dioscoridi.lorenzo@virgilio.it

Abstract

Pure endoscopic treatment of combined cholelithiasis and choledocholithiasis is possible due to the chance to use together both endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS) approaches. This endotherapy permits to treat biliary stones in the main bile duct by standard ERCP and gallbladder stones by EUS-guided cholecystoduodenostomy eventually associated to intracorporeal lithotripsy to achieve optimal results.

Key Words: Endoscopic ultrasound-guided gallbladder drainage; Biliary lithotripsy; Gallstone lithotripsy; Gallbladder biliary lithotripsy; New rationale

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Core Tip: Combining endoscopic approaches of endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatography (ERCP) let to treat simultaneously biliary stones both in the gallbladder and in the main bile ducts. ERCP standard approach can be associated to EUS-guided gallbladder drainage to avoid recurrences in patients unfit for surgery. To optimize this treatment, gallbladder stones can be fragmented by intracorporeal lithotripsy so their fragments can easily pass through the stent in place for cholecystoduodenostomy. This mininvasive approach seems promising on the base of available literature.

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TO THE EDITOR

We read with interest the paper by Cianci and Restini[1].

The authors interestingly discussed on indications, advantages and disadvantages of laparo-endoscopic rendez-vous, pure laparoscopic common bile duct exploration and sequential laparoscopic-endoscopic retrograde cholangiopancreatography (ERCP) approach in combined cholethiasis and choledocholithiasis.

We would like to focus on a totally endoscopic approach to this pathology.

We totally agree with Cianci and Restini[1] that ERCP cannot be considered definitive in the treatment of cholecysto-choledocholithiasis, even if associated with lithotripsy, since the genesis of the stones is secondary to the lithogenic bile in the gallbladder. Thus, can we act endoscopically also on gallbladder stones?

Recently, EUS-guided cholecystoduodenostomy was found comparable to laparoscopic cholecystectomy in terms of clinical outcomes[2]. Recurrent biliary events after EUS-guided gallbladder drainage can be even lowered by associated lithotripsy.

Historically, many studies demonstrated the ineffectiveness of the gallstone lithotripsy alone with high recurrence rate associated to high rate of adverse events, especially in case of multiple and large stones[3,4].

In the recent years, the role of this treatment was re-established because of the new endoscopic approaches described for acute cholecystitis; particularly, the increasing role of EUS-guided cholecysto-duodenostomy[5,6] has two main interesting implications in this perspective.

Firstly, EUS-guided gallbladder drainage can be considered as definitive therapy especially in elderly patients unfit for surgery, differently from percutaneous cholecystostomy[4]. After endoscopic stable gallbladder drainage in the duodenum, the gallbladder loses its property to concentrate the bile and, so on, to form new gallstones.

In this setting, the opportunity to destroy the gallstones is interesting, on one hand, to avoid eventually obstruction of the biliary edge of the stent (and subsequent relapses of symptoms) or, on the other hand, further migration of biliary gallstones into the infundibulum or into the biliary tree (and subsequent obstruction of the main bile duct), especially in case of hard and large stones.

Intracholecystic lithotripsy can be theoretically performed as biliary standard lithotripsy because effectiveness and adverse events should not be different, as described in few first experiences[6-8]. On the other side, standard endoscopic treatment for lithiasis of the common bile duct can be provided in the same combined session.

The main limitations of EUS-guided gallbladder drainage are still the need of a sufficient loosening of the gallbladder (evaluated at the preliminary EUS) to guarantee a safe puncture and the absence of extraluminal pericholecystic fluid collection.

Moreover, intracorporeal lithotripsy in the setting acute cholecystitis can be associated with higher risk of perforation because of the acute inflammation of the gallbladder walls[7-9].

We encourage further studies on this focus to verify these first results and to improve the outcomes and indications of endoscopic treatments in this field, especially for patients unfit for surgery.

FOOTNOTES

Author contributions: Dioscoridi L and Mutignani M designed research; Dioscoridi L wrote the letter; Mutignani M revised the letter.

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Country/Territory of origin: Italy

ORCID number: Lorenzo Dioscoridi 0000-0003-4975-018X; Massimiliano Mutignani 0000-0002-1272-4888.

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LETTER TO THE EDITOR

Gastrointestinal microbiome and *Helicobacter pylori*: Eradicate, leave it as it is, or take a personalized benefit–risk approach?

Stanislav Sitkin, Leonid Lazebnik, Elena Avalueva, Svetlana Kononova, Timur Vakhitov

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Stanislav Sitkin, Elena Avalueva, Department of Internal Diseases, Gastroenterology and Dietetics, North-Western State Medical University Named After I.I. Mechnikov, St. Petersburg 191015, Russia

Stanislav Sitkin, Svetlana Kononova, Timur Vakhitov, Non-Infectious Disease Metabolomics Group, Institute of Experimental Medicine, St. Petersburg 197376, Russia

Stanislav Sitkin, Epigenetics and Metagenomics Group, Institute of Perinatology and Pediatrics, Almazov National Medical Research Centre, St. Petersburg 197341, Russia

Leonid Lazebnik, Department of Outpatient Therapy, A.I. Yevdokimov Moscow State University of Medicine and Dentistry, Moscow 127473, Russia

Svetlana Kononova, Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow region 142290, Russia

Corresponding author: Stanislav Sitkin, MD, PhD, Associate Professor, Senior Researcher, Department of Internal Diseases, Gastroenterology and Dietetics, North-Western State Medical University named after I.I. Mechnikov, Kirochnaya Street, 41, St. Petersburg 191015, Russia. drsitkin@gmail.com

Abstract

Helicobacter pylori (*H. pylori*) is generally regarded as a human pathogen and a class 1 carcinogen, etiologically related to gastric and duodenal ulcers, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. However, *H. pylori* can also be regarded as a commensal symbiont. Unlike other pathogenic/opportunistic bacteria, *H. pylori* colonization in infancy is facilitated by T helper type 2 immunity and leads to the development of immune tolerance. Fucosylated gastric mucin glycans, which are an important part of the innate and adaptive immune system, mediate the adhesion of *H. pylori* to the surface of the gastric epithelium, contributing to successful colonization. *H. pylori* may have beneficial effects on the host by regulating gastrointestinal (GI) microbiota and protecting against some allergic and autoimmune disorders and inflammatory bowel disease. The potential protective role against inflammatory bowel disease may be related to both modulation of the gut microbiota and the immunomodulatory properties of *H. pylori*. The inverse association between *H. pylori* and some potentially proinflammatory and/or procarcinogenic bacteria may suggest it regulates the GI microbiota. Eradication of *H. pylori* can cause various adverse effects and alter the GI microbiota, leading to short-term or long-term dysbiosis.

Overall, studies have shown that gastric Actinobacteria decrease after *H. pylori* eradication, Proteobacteria increase during short-term follow-up and then return to baseline levels, and Enterobacteriaceae and *Enterococcus* increase in the short-term and interim follow-up. Various gastric mucosal bacteria (*Actinomyces*, *Granulicatella*, *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Rothia*, *Streptococcus*, *Rhodococcus*, and *Lactobacillus*) may contribute to precancerous gastric lesions and cancer itself after *H. pylori* eradication. *H. pylori* eradication can also lead to dysbiosis of the gut microbiota, with increased Proteobacteria and decreased Bacteroidetes and Actinobacteria. The increase in gut Proteobacteria may contribute to adverse effects during and after eradication. The decrease in Actinobacteria, which are pivotal in the maintenance of gut homeostasis, can persist for > 6 mo after *H. pylori* eradication. Furthermore, *H. pylori* eradication can alter the metabolism of gastric and intestinal bacteria. Given the available data, eradication cannot be an unconditional recommendation in every case of *H. pylori* infection, and the decision to eradicate *H. pylori* should be based on an assessment of the benefit–risk ratio for the individual patient. Thus, the current guidelines based on the unconditional "test-and-treat" strategy should be revised. The most cautious and careful approach should be taken in elderly patients with multiple eradication failures since repeated eradication can cause antibiotic-associated diarrhea, including severe *Clostridioides difficile*-associated diarrhea and colitis and antibiotic-associated hemorrhagic colitis due to *Klebsiella oxytoca*. Furthermore, since eradication therapy with antibiotics and proton pump inhibitors can lead to serious adverse effects and/or dysbiosis of the GI microbiota, supplementation of probiotics, prebiotics, and microbial metabolites (*e.g.*, butyrate + inulin) should be considered to decrease the negative effects of eradication.

Key Words: *Helicobacter pylori*; Eradication; Gastrointestinal microbiota; Dysbiosis; Fucosylated glycan; Inflammatory bowel disease

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Core Tip: *Helicobacter pylori* (*H. pylori*) is generally regarded as a human pathogen, but it can act as a commensal symbiont. *H. pylori* colonization may have beneficial effects on the host by regulating gastrointestinal microbiota and protecting against some allergic and autoimmune disorders and inflammatory bowel disease. *H. pylori* eradication can cause various adverse effects and alter the gastrointestinal microbiota, leading to dysbiosis. Therefore, eradication cannot be an unconditional recommendation in every case of *H. pylori* infection, and the therapeutic decision should be based on a personalized assessment of the benefit *vs* risk.

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TO THE EDITOR

We read with great interest the article by Niu *et al*[1], which showed that the effectiveness of quadruple *Helicobacter pylori* (*H. pylori*) eradication therapy containing bismuth depended on the gastric microbiota, and a high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*, which were significantly enriched in the gastric mucosa in the successful eradication group[1]. The role of lactobacilli, mainly beneficial bacteria, in *H. pylori* infection, including eradication, has been well studied[2]. However, the authors showed for the first time the importance of *Rhodococcus* and *Sphingomonas*, which are more likely to be opportunistic or pathobiont species with unclear functions in the human gastrointestinal (GI) tract[3,4], in successful eradication of *H. pylori*[1]. It is noteworthy that in gastric cancer (GC), when the abundance of *H. pylori* decreased, several taxa (including *Rhodococcus* and *Lactobacillus*, discussed by Niu *et al*[1]) in the gastric mucosa significantly increased, which may indicate their potential involvement in GC after *H. pylori* infection[5]. In addition, *H. pylori* was negatively correlated with some opportunistic bacteria/pathobionts such as *Haemophilus*, *Streptococcus*, *Neisseria*, and *Fusobacterium* in the success group[1]. The results obtained by Niu *et al*[1] may suggest that *H. pylori* competes not so much with beneficial bacteria as with pathobionts, and eradication may ultimately worsen the gastric microbiota.

Indeed, not only does the composition of the gastric microbiota affect *H. pylori* eradication, but eradication significantly affects the microbiota of both the stomach and intestine, which can lead to marked and long-term dysbiotic changes. Dysbiosis of the microbiota after *H. pylori* eradication can be caused by many factors: by the action of antibiotics and proton pump inhibitors; by the loss of *H. pylori* leading to changes in the immune response of the GI mucosa; and by changes in the microenvironment of the GI tract, including those in microbial metabolic pathways and changes in gastric acidity associated with both pharmacotherapy and loss of *H. pylori*[6,7]. Features of the dysbiotic changes, their duration, and the rate of restoration of the disturbed microbiota vary greatly in different studies.

Diverse effects of H. pylori eradication on gastric and intestinal microbiota

Recent studies have demonstrated not only short-term but also long-term (≥ 6 mo) changes in the gastric microbiota after *H. pylori* eradication. In about half of cases (52.3%), eradication led to the predominance of proinflammatory *Acinetobacter* in gastric corpus mucosa with a decrease in microbial diversity in patients with endoscopic follow-up for > 1 year[8]. An earlier study showed that *Acinetobacter* was enriched in patients with persistent gastric inflammation 1 year after *H. pylori* eradication[9]. Moreover, some bacteria in the gastric mucosa (*Actinomyces*, *Granulicatella*, *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Rothia*, and *Streptococcus*), which are predominantly of oral origin, were associated with precancerous gastric lesions (atrophy and/or intestinal metaplasia) 1 year after *H. pylori* eradication[9]. *Actinomyces*, whose abundance can increase in the absence of *H. pylori*, might significantly increase the risk of GC [10]. Thus, some studies demonstrated a contribution of various gastric bacteria to precancerous gastric lesions after *H. pylori* eradication[9]. In general, Actinobacteria decreased after *H. pylori* eradication, Proteobacteria increased during short-term follow-up and then returned to baseline levels, and Enterobacteriaceae and *Enterococcus* increased in the short-term and interim follow-up[11]. Alternatively, it has been shown that in regions with high GC risk, *H. pylori* is one of the main factors in gastric dysbiosis and successful eradication can lead to the restoration of gastric microbiota[12].

H. pylori eradication also affects the gut microbiota. Bismuth quadruple therapy leads to short-term dysbiosis of the gut microbiota with an increased abundance of Proteobacteria and decreased abundances of Bacteroidetes and Actinobacteria. The increase in gut Proteobacteria may contribute to adverse effects during eradication therapy[13]. In another study, *H. pylori* eradication was associated with significant alterations in the gut microbiota that did not completely recover 6 wk after treatment [7]. In general, there was a decrease in Actinobacteria, which are pivotal in the maintenance of gut homeostasis, compared with baseline throughout the follow-up (> 6 mo) after eradication[11]. Furthermore, eradication therapy alters microbial functional pathways and the metabolism of gastric and gut bacteria[9,14].

Conversely, other studies showed that successful *H. pylori* eradication exerts beneficial effects on gut microbiota, including increased probiotic *Bifidobacterium* and downregulation of drug-resistance mechanisms[12]. Liou *et al*[15] generally confirmed the long-term safety of *H. pylori* eradication therapy but reported incomplete restoration of microbial diversity after 1 year and clinically irrelevant but significant increases in body mass index (BMI) and body weight at that time.

Interestingly, an increase in body weight/body mass index after *H. pylori* eradication had been identified earlier[16]. Suggested mechanisms of this effect range from an improvement in the symptoms of postprandial dyspepsia[16] to changes in the regulation of leptin and ghrelin[17] mediated by antibiotic-associated changes in the microbiota (especially by the imbalance between bacterial producers of lactate and acetate)[18]. In general, however, the data in various studies are contradictory and indicate weight gain, weight loss, or the absence of an effect of *H. pylori* eradication on body weight; this may be due to differences in the characteristics of the studied populations, such as age, nosology, and composition of the GI microbiota[19]. Further in-depth study of the microbiome-mediated effects of *H. pylori* and eradication therapy on human host metabolism, including nutrient uptake, energy homeostasis, bodyweight, hormone secretion, lipid profile, and glucose homeostasis/glycemic control, will provide clinically important findings for the management of *H. pylori* infection.

H. pylori status and the human gut microbiome

The presence or absence of gastric *H. pylori* can significantly affect the gut microbiota. For example, Nitrospirae were found exclusively in *H. pylori*-negative patients. The role of this phylum, containing nitrite-oxidizing bacteria, in the human microbiome is unclear. In a study by Wang *et al*[20], Nitrospirae were found in the gastric mucosa in all patients with GC but not in patients with chronic gastritis. The authors suggested that these bacteria may be involved in carcinogenesis through enhanced production of N-nitroso compounds. A recent study demonstrated a possible pathogenetic link between enriched colonic Nitrospirae and drug-resistant epilepsy, implying that Nitrospirae can increase nitrite toxicity and cause blood-brain barrier dysfunction[21].

Proinflammatory *Bacteroides ovatus* and *Fusobacterium varium*, associated with ulcerative colitis and adenomatous polyps[22,23] as well as trimethylamine-producing *Clostridium* sp. AT5[24] were enriched in *H. pylori*-negative samples, while *Bacteroides plebeius*, characteristic of the healthy groups (*vs* patients with adenomatous polyps)[23] and butyrate-producing *Eubacterium ramulus* were enriched in *H. pylori*-positive samples[7].

Conversely, *Butyricimonas* spp., including *Butyricimonas virosa*, associated with bacteremia in patients with GI cancer (colon and duodenal adenocarcinomas) and diverticulitis[25] as well as *Bacteroides coprophilus*, specifically enriched in ankylosing spondylitis[26], were enriched in *H. pylori*-positive individuals[7]. Proinflammatory *Prevotella copri*, associated with rheumatoid arthritis and microscopic colitis as well as *Enterobacter cloacae* and *Klebsiella pneumoniae*, pathogens commonly associated with hospital infections, were also enriched in *H. pylori*-positive individuals[27]. Moreover, gut microbial vitamin B_{12} biosynthesis was significantly lower in *H. pylori*-positive individuals compared with *H. pylori* -negative individuals[27]. Dash *et al*[28] showed that the gut microbiota of *H. pylori*-infected individuals was characterized by a significantly increased abundance of *Succinivibrio*, Coriobacteriaceae, Enterococcaceae and Rikenellaceae as well as *Candida glabrata* and other unclassified fungi. The authors suggested a possible role for these *H. pylori*-associated changes in the gut microbiota in intestinal barrier disruption and development of colorectal carcinoma[28].

Potential protective and regulatory role of commensal H. pylori

Currently, *H. pylori* is generally regarded as a human pathogen and a class 1 carcinogen[29], responsible for 15% of the total cancer burden globally and up to 89% of all GC cases[30]. *H. pylori* is etiologically related to gastric and duodenal ulcers and mucosa-associated lymphoid tissue lymphoma. According to current guidelines, it is almost always subject to unconditional eradication based on the "test-and-treat" strategy[31]. However, although *H. pylori* is present in > 50% of the world's population, sequelae of infection occur in only 20% of infected individuals, and malignant complications, such as GC, occur in < 3% of infected people[7]. Therefore, there is also an alternative point of view that *H. pylori* is a commensal symbiont[32,33]. Back in 1998, Blaser[32] wrote that, "*H. pylori* can thus be regarded as indigenous or 'normal' flora, which most humans acquire within the first few years of childhood and then carry for life."

Unlike other pathogenic/opportunistic bacteria, *H. pylori* colonization of newborns/infants is facilitated by T helper type 2 immunity and leads to the development of immune tolerance[34]. Most likely, the co-evolution of *H. pylori* and the human host over millennia has led to the fact that this bacterium is considered a commensal symbiont, not a pathogen, by the host's immune system. This is indirectly confirmed by the fact that α1,2-fucosylated glycans of the GI epithelium, which are an important part of the innate and adaptive immune system (they create a symbiotic environment for the host and microbiota and protect against pathogens), mediate the adhesion of *H. pylori* to the surface of the gastric epithelium, contributing to successful colonization[35]. As a result, early colonization of *H. pylori* can have a positive effect on the host, for example on the regulation of the hormones leptin and ghrelin as well as on protection against some allergic (*e.g.*, asthma) and autoimmune diseases and against inflammatory bowel disease (IBD)[36,34]. The inverse association between *H. pylori* and some potentially proinflammatory and/or procarcinogenic bacteria found in various studies[20,22-24] may suggest a regulatory function of *H. pylori* toward the GI microbiota. The available evidence for beneficial effects of *H. pylori* toward the GI microbiota, as well as potential protective effects against certain diseases, should not be ignored by the gastroenterological community.

Presence of *H. pylori* infection and reduced risk of IBD: Is there a causal relationship? The potential protective role of *H. pylori* in the development of IBD, shown in some studies[37], may be related to both modulation of the gut microbiota and the immunomodulatory properties of *H. pylori*. An inverse association between *H. pylori* and potentially proinflammatory microbes (*Bacteroides ovatus*, *Fusobacterium varium*, *Rhodococcus*, *Sphingomonas*) supports a microbiome-modulating mechanism, while an immunomodulatory mechanism of protection against IBD may involve the activation of colonic mucus production by *H. pylori via* the NLRP3/caspase-1/interleukin-18 axis[38].

We suggest another possibility for the association between *H. pylori* infection and IBD, a non-causal relationship, which relates to the fucosylation status of host mucin glycans in the GIT. Individuals with a non-functional α(1,2)-fucosyltransferase 2 (*FUT2*) gene (they are termed non-secretors), who have loss-of-function mutations, cannot express α(1,2)-fucosylated glycans in the GI mucosa. Non-secretors (about 20% of the population) are more susceptible to infection by some pathogens (*Escherichia coli*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Candida albicans*) and have aberrant gut microbiota, with a reduction of beneficial *Bifidobacterium* spp. The *FUT2* non-secretor phenotype increases susceptibility to Crohn's disease, ulcerative colitis, primary sclerosing cholangitis, celiac disease, psoriasis, Behçet's disease, type 1 diabetes, and so on but at the same time protects against *H. pylori*, which requires fucosylated glycans in the gastric mucosa for adhesion[35,39,40].

A recent study showed that patients with ulcerative colitis and Crohn's disease had decreased *FUT2* expression and α1,2-fucosylation in the colon[41]. In addition, *Fut2* deficiency in the intestinal epithelium exacerbated colitis in epithelium-specific *Fut2* knockout ($Fut2^{\Delta IEC}$) mice, promoted the release of proinflammatory cytokines, and aggravated epithelial barrier damage[41]. The authors demonstrated for the first time that epithelium-specific *Fut2* deficiency increased susceptibility to IBD through modulation of the gut microbiome and microbiota-mediated lysophosphatidylcholine generation. Lysophosphatidylcholine may have deleterious effects on the colon by promoting the release of proinflammatory cytokines, damaging the tight junctions and epithelial barrier in the colon epithelium, and exacerbating colonic inflammation in $Fut2^{\Delta IEC}$ mice[41].

In turn, upregulation of *FUT2*-mediated fucosylation in the intestinal epithelium, for example by exogenous L-fucose, can protect the intestinal barrier, enhance tight junctions, and alleviate intestinal inflammation[42]. Similar to the colon, increased expression of the fucosyltransferase genes *FUT2* and *FUT1* in the gastric epithelium promotes *H. pylori* adhesion and ultimately infection[43]. Thus, we suggest that increased expression of *FUT2* in the gastrointestinal mucosa can simultaneously mediate both *H. pylori* infection and protection against IBD *via* an *H. pylori*-independent mechanism. In this case, a causal relationship between the presence of *H. pylori* and a reduced risk of IBD is unlikely. However, it cannot be ruled out that *H. pylori* mediates the protective effect of fucosylation against IBD by modulation of the gut microbiota or through an immunomodulatory mechanism[38].

Concluding remarks

Given the available data, eradication cannot be an unconditional recommendation in every case of *H. pylori* infection, as in the vast majority of people *H. pylori* is most likely a commensal[32,33], possibly beneficial in mutualistic interaction with the host (for example protecting against some allergic and autoimmune diseases[36]). We join the opinion of Chen *et al*[7] that the decision to eradicate *H. pylori* should be based on an assessment of the benefit–risk ratio for the individual patient. We also support the position of Miller and Williams[44] that "universal eradication" of *H. pylori* may cause more harm than good for the infected persons. Thus, the current guidelines based on the unconditional "test-and-treat" strategy[31] should be revised, including to reduce the excessive number of indications for eradication and to avoid empirical eradication therapy without a previous diagnostic test for *H. pylori* infection.

It may be worth recommending unconditional eradication only in patients with concomitant mucosa-associated lymphoid tissue lymphoma[45] and/or in individuals at high risk of GC, for example in groups of high familial (hereditary) risk[46] or in high-risk areas/populations where eradication effectively reduces the risk of GC[47]. In the latter case, the advisability of such an approach is unquestionable, if it is evidence-based. For example, a recent systematic review and meta-analysis provided moderate-certainty evidence that searching for and eradicating *H. pylori* can reduce the subsequent incidence of GC and death from GC in healthy asymptomatic infected people; the risk of GC decreased by 46% after eradication therapy[48]. However, the authors concluded that as all but one of the eligible trials were conducted in East Asian populations (in China, Japan, or South Korea), and the only trial conducted in a non-Asian population (in Colombia) did not demonstrate any benefit of such an approach, the results of the systematic review cannot be extrapolated to populations outside East Asia [48].

A well-known paradox, the low incidence of GC in some regions of Africa, Asia (*e.g.*, in India), and Latin America with a high prevalence of *H. pylori* infection, also requires in-depth study; this is called the African[49] or Asian/Indian enigma[50]. Although the existence of this phenomenon is sometimes disputed[51], studies have shown that *H. pylori* alone is most likely not enough for the development of GC, even with a high prevalence of highly pathogenic strains. Therefore, it is necessary to take into account not only the virulence factors of *H. pylori* and the oncogenic potential of specific strains of *H. pylori* but also the genetics and ethnicity of the human host population, their dietary habits (including antioxidant and sodium levels), smoking, alcohol consumption, socioeconomic status, and coinfection (parasitoses/helminthiases) modulating the potentially protective T helper type 2 immune response[49,50].

An important factor influencing the serious consequences of *H. pylori* infection appears to be the co-evolution of *H. pylori* and the human host. A recent study demonstrated that the African human ancestry showed clear signs of co-evolution with *H. pylori*, while the European ancestry was maladapted. The Asian ancestry was intermediate but closer to the African ancestry[52]. This supports the hypothesis that *H. pylori* is a commensal symbiont rather than a human pathogen. Hopefully, a series of international prevalence surveys to investigate age-specific prevalence of *H. pylori* in areas of low and high GC risk, namely ENIGMA, recently launched under the auspices of the International Agency for Research on Cancer in Africa (Uganda), Asia (Iran), and Latin America (Chile, Costa Rica), will shed light on the regional characteristics of *H. pylori* infection and identify markers for GC risk stratification to offer reasonable preventive interventions for different populations[53].

In addition, *H. pylori* eradication is likely to be recommended in patients with cancer who are on therapy with immune checkpoint inhibitors or vaccine-based immunotherapy, for example in patients with non-small-cell lung cancer[54].

In patients with diseases negatively associated with *H. pylori*, such as IBD, microscopic colitis, celiac disease, asthma, multiple sclerosis, Barrett's esophagus, esophageal adenocarcinoma, eosinophilic esophagitis, and so on, eradication should be carried out with caution, carefully weighing the risk-to-benefit in each case. Even though *H. pylori* eradication did not affect either the healing rate or the recurrence rate of pre-existing gastroesophageal reflux disease, the possibility of developing new erosive gastroesophageal reflux disease after eradication should always be kept in mind[55].

The most cautious and careful approach should be taken in elderly patients with multiple eradication failures since repeated eradication (second-/third-line therapies) can cause antibiotic-associated diarrhea, including severe *Clostridioides difficile*-associated diarrhea and colitis[56] and antibiotic-associated hemorrhagic colitis due to *Klebsiella oxytoca*[57]. In this regard, we support the recent

conclusion of the American Gastroenterological Association experts that, "after multiple failed eradication attempts, the potential benefits of *H. pylori* eradication should be weighed carefully against the likelihood of adverse effects and inconvenience of repeated high-dose acid suppression and antibiotic exposure, particularly among individuals who are not at an identifiably higher risk of complications from persistent *H. pylori* infection (*e.g.*, GC, peptic ulcer disease); in such scenarios, a shared decision-making approach should be seriously considered, especially in the elderly, those with frailty, and those with intolerance to antibiotics" (Best Practice Advice #9)[30].

Furthermore, since eradication therapy with antibiotics and proton pump inhibitors can lead to serious adverse effects and/or long-term dysbiotic changes in the GI microbiota, the supplementation of probiotics[58-61], prebiotics, and microbial metabolites (*e.g.*, butyrate + inulin)[62] to reduce the negative effects of eradication should be considered[7]. In addition, alternative eradication regimens with limited or no antibiotic use, for example phage-based regimens[63], autoprobiotics[64], and natural agents and methods including traditional Chinese medicine[65], should be proposed, developed, and explored in future studies.

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Country/Territory of origin: Russia

ORCID number: Stanislav Sitkin 0000-0003-0331-0963; Leonid Lazebnik 0000-0001-8736-5851; Elena Avalueva 0000-0001-6011-0998; Svetlana Kononova 0000-0002-7373-7797; Timur Vakhitov 0000-0001-8221-6910.

Corresponding Author's Membership in Professional Societies: European Crohn's and Colitis Organisation (ECCO), Member ID: 37495.

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Contents

Weekly Volume 28 Number 8 February 28, 2022

REVIEW

775 Clinical implications of diabetes in chronic liver disease: Diagnosis, outcomes and management, current and future perspectives

García-Compeán D, Orsi E, Kumar R, Gundling F, Nishida T, Villarreal-Pérez JZ, Del Cueto-Aguilera ÁN, González-González JA, Pugliese G

MINIREVIEWS

794 Mixed neuroendocrine–nonneuroendocrine neoplasms of the gastrointestinal system: An update

Elpek GO

ORIGINAL ARTICLE

Basic Study

811 Mucosal bacterial dysbiosis in patients with nodular lymphoid hyperplasia in the terminal ileum

Jiang QL, Lu Y, Zhang MJ, Cui ZY, Pei ZM, Li WH, Lu LG, Wang JJ, Lu YY

Retrospective Cohort Study

825 Differential DNA methylation analysis of *SUMF2*, *ADAMTS5*, and *PXDN* provides novel insights into colorectal cancer prognosis prediction in Taiwan

Su JQ, Lai PY, Hu PH, Hu JM, Chang PK, Chen CY, Wu JJ, Lin YJ, Sun CA, Yang T, Hsu CH, Lin HC, Chou YC

Retrospective Study

840 Long-term outcomes of endoscopic submucosal dissection and surgery for undifferentiated intramucosal gastric cancer regardless of size

Lee GH, Lee E, Park B, Roh J, Lim SG, Shin SJ, Lee KM, Noh CK

853 Inverse correlation between gastroesophageal reflux disease and atrophic gastritis assessed by endoscopy and serology

Han YM, Chung SJ, Yoo S, Yang JI, Choi JM, Lee J, Kim JS

CASE REPORT

868 Treatment strategy for pancreatic head cancer with celiac axis stenosis in pancreaticoduodenectomy: A case report and review of literature

Yoshida E, Kimura Y, Kyuno T, Kawagishi R, Sato K, Kono T, Chiba T, Kimura T, Yonezawa H, Funato O, Kobayashi M, Murakami K, Takagane A, Takemasa I

LETTER TO THE EDITOR

878 "Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel disease": A letter to the editor

Mc Gettigan N, O'Toole A, Boland K

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Weekly Volume 28 Number 8 February 28, 2022

Contents

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REVIEW

Clinical implications of diabetes in chronic liver disease: Diagnosis, outcomes and management, current and future perspectives

Diego García-Compeán, Emanuela Orsi, Ramesh Kumar, Felix Gundling, Tsutomu Nishida, Jesús Zacarías Villarreal-Pérez, Ángel N Del Cueto-Aguilera, José A González-González, Giuseppe Pugliese

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Diego García-Compeán, Gastroenterology Service and Department of Internal Medicine, Faculty of Medicine, University Hospital "Dr. José E. González", Universidad Autónoma de Nuevo Le ón, Monterrey 64700, Nuevo León, Mexico

Emanuela Orsi, Diabetes Service, Endocrinology and Metabolic Diseases Unit, Fdn IRCCS Ca Granda, Endocrine Unit, Padigl Granelli, Milan 20121, Italy

Ramesh Kumar, Department of Gastroenterology, All India Institute of Medical Sciences, Patna 801507, India

Felix Gundling, Department of Gastroenterology, Gastrointestinal Oncology, Hepatology, Diabetics, Metabolism and Infectious Diseases, Sozialstiftung Bamberg, Bamberg 96049, Germany

Tsutomu Nishida, Department of Gastroenterology, Toyonaka Municipal Hospital, Osaka 560-8565, Japan

Jesús Zacarías Villarreal-Pérez, Department of Endocrinology, University Hospital, Autonomous University of Nuevo León, Monterrey 64700, Mexico

Ángel N Del Cueto-Aguilera, Department of Gastroenterology and Internal Medicine, Faculty of Medicine, University Hospital, Autonomous University of Nuevo León, Monterrey 64700, Nuevo León, Mexico

José A González-González, Gastroenterology Service and Department of Internal Medicine, University Hospital Dr. José E González and Medical School, Monterrey 64460, Nuevo León, Mexico

Giuseppe Pugliese, Department of Clinical and Molecular Medicine, La Sapienza University, Roma 00161, Italy

Corresponding author: Diego García-Compeán, MD, MSc, Professor, Gastroenterology Service and Department of Internal Medicine, Faculty of Medicine, University Hospital "Dr. José E. González", Universidad Autónoma de Nuevo León, Madero y Gonzalitos S/N, Monterrey 64700, Nuevo León, Mexico. digarciacompean@prodigy.net.mx

Abstract

Diabetes mellitus (DM) is common in liver cirrhosis (LC). The pathophysiological association is bidirectional. DM is a risk factor of LC and LC is a diabetogenic

condition. In the recent years, research on different aspects of the association DM and LC has been intensified. Nevertheless, it has been insufficient and still exist many gaps. The aims of this review are: (1) To discuss the latest understandings of the association of DM and LC in order to identify the strategies of early diagnosis; (2) To evaluate the impact of DM on outcomes of LC patients; and (3) To select the most adequate management benefiting the two conditions. Literature searches were conducted using PubMed, Ovid and Scopus engines for DM and LC, diagnosis, outcomes and management. The authors also provided insight from their own published experience. Based on the published studies, two types of DM associated with LC have emerged: Type 2 DM (T2DM) and hepatogenous diabetes (HD). High-quality evidences have determined that T2DM or HD significantly increase complications and death pre and post-liver transplantation. HD has been poorly studied and has not been recognized as a complication of LC. The management of DM in LC patients continues to be difficult and should be based on drug pharmacokinetics and the degree of liver failure. In conclusion, the clinical impact of DM in outcomes of LC patients has been the most studied item recently. Nevertheless many gaps still exist particularly in the management. These most important gaps were highlighted in order to propose future lines for research.

Key Words: Diabetes mellitus; Liver cirrhosis; Hepatogenous diabetes; Clinical implications; Therapy

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Core Tip: The prevalence of diabetes mellitus (DM) and impaired glucose tolerance in patients with liver cirrhosis (LC) is around 30% and 40% respectively. DM is a risk factor for LC and LC is a diabetogenic condition. Two types of diabetes associated with LC have emerged: Type 2 DM and hepatogenous diabetes (HD). However HD has not been recognized as a complication of LC. It is widely accepted that DM increases complications and mortality in cirrhotic patients. DM treatment is quite difficult due to liver failure. In the present review we will discuss the most recent information published in this field, pointing out the gaps that still exist in the subject.

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INTRODUCTION

For some time, type 2 diabetes mellitus (T2DM) has been suggested as a risk factor for chronic liver disease (CLD)[1]. Besides, the diabetogenic nature of liver cirrhosis (LC) was described and the term "hepatogenous diabetes" (HD) was coined hepatogenous diabetes (HD) in order to differentiate it from T2DM and attributed it to hepatic dysfunction[2].

In 1994, Bianchi *et al*[3] demonstrated for the first time the negative predictive capability of DM on the outcome of LC patients by observing a significant increase in mortality due to liver complications. Up to date, it is known that DM and impaired glucose tolerance (IGT) are common in LC patients worldwide, particularly in this era of nonalcoholic fatty liver disease (NAFLD) and obesity. From the beginning of this millennium, research on the different aspects of the association between DM and LC has been progressively intensified giving rise to important results. Therefore, we decided to critically review this scientific evidence generated so far highlighting the gaps that still exist in this complex field in order to propose future lines for research.

We also attempt (based on available evidence) to catalyze the recognition of HD as an entity with its own epidemiologic, physiopathological, and clinical implications for patients with LC, creating a template for future refinement of this condition.

EPIDEMIOLOGY

Between 20% and 60% of patients with LC may have overt DM, from 60% to 80% may have IGT and close to 100% insulin resistance (IR)[4,5]. The prevalence of impaired glucose homeostasis seems to be

increased by the severity of CLD. The prevalence of T2DM and HD in compensated LC patients has been determined in 19.2% and 21.5%, respectively[6]. NAFLD, malnutrition, alcohol abuse, hepatitis C virus (HCV) or hepatitis B virus (HBV) infection and primary hemochromatosis are associated to an increased risk of HD[7]. In 2000, the National Health and Nutrition Examination Survey carried out in United States, demonstrated a 3-fold increased prevalence of DM in HCV carriers compared with the average population[8]. In other studies, the extent of fibrosis in patients with hepatitis B and C correlated with an increased prevalence of DM[9,10].

Many clinical studies have shown that DM may increase the severity and accelerate the progression of liver failure leading to a significant increase in liver complications and mortality[4,11]. In the other side, HD has been found to have well defined clinical and pathophysiological characteristics that allow to differentiate it from T2DM[12,13]. HD can be suspected in LC patients without personal or family history of DM and metabolic syndrome (MS)[12].

Notwithstanding, the conceptual term "hepatogenous diabetes" is not included in the currently valid national and international classification systems describing etiologies of DM[14], neither HD is accepted as a complication of LC by the World Health Organization (WHO). Maybe for this reason, the diagnosis and treatment of IGT and DM in LC patients are often overlooked by physicians as was shown by a questionnaire applied to 576 gastroenterologists in Germany. The 90% and 40% of physicians underestimated the prevalence of IGT and DM in cirrhotic patients respectively[15]. In another study, it was found that, in contrast to other complications associated with cirrhosis, HD was underestimated even among medical staff from highly specialized hospital departments[16].

PATHOPHYSIOLOGY

The pathophysiological relationship between DM and LC is bidirectional. In one side, T2DM may lead to liver disease in the context of the MS and NAFLD and, in the other side, LC is a diabetogenic condition[17,18]. The pathophysiology of liver disease due to T2DM (NAFLD) is not discussed in this text as it can be reviewed elsewhere. In contrast, liver failure, portosystemic shunts, hyperinsulinemia, increased glucagon, growth hormone, insulin-like growth factor, free fatty acids and cytokines that induce peripheral IR and β-cells dysfunction play a significant pathogenic role in HD[17,18] (Figure 1).

IR and hyperinsulinemia

The liver plays a key role in glucose metabolism as the major site of glycogen synthesis and gluconeogenesis. Hepatocellular functional impairment results in abnormal glycogen synthesis and decreased hepatic capacity for glycogen deposits[13]. IR in peripheral tissues (adipose and muscular tissues) and liver dysfunction play a central role in the glucose metabolism disturbance[19,20]. Reduced insulin clearance by the damaged liver and portosystemic shunts result in hyperinsulinemia which is potentiated by raised levels of contra-insulin hormones (glucagon, growth hormone, insulin-like growth factor) and free fatty acids and cytokines[20,21].

Hyperinsulinemia can be detected in the early stages of CLD, both in the fasting and postprandial state. A major precipitating factor of hyperinsulinemia is the reactive insulin hypersecretion by the pancreas for compensation of peripheral IR in muscle tissue and impaired hepatic glucose utilization.

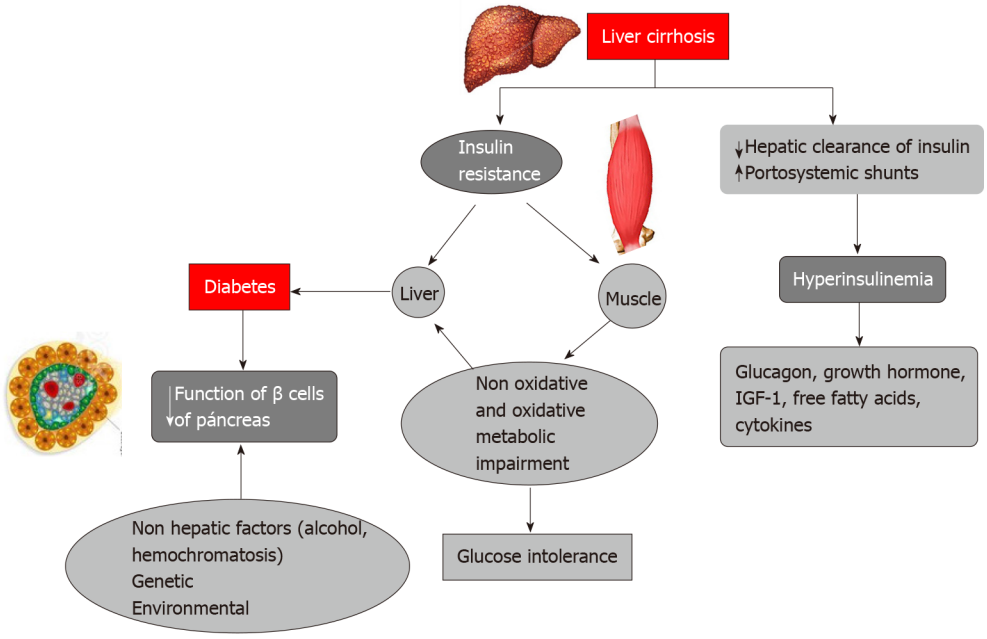
Pancreatic beta cells adaptation

Inadequate early increase in insulin secretion and decreased hepatic glucose production are often observed in LC even in the absence of DM. The progressive loss of insulin secretion culminates in a step-wise fashion in DM[22]. The trigger seems to be glucotoxicity from chronic hyperglycemia, which causes secretory impairment of pancreatic β-cells[23].

NAFLD AND DM

NAFLD is the most common CLD in the world and its prevalence in the general population is between 17% and 46%. NAFLD is closely related to MS and DM. For this reason an international consensus panel of experts have recommended the redefinition of this disease with the term of metabolism associated fatty liver disease[24,25]. Notwithstanding, NAFLD may also affect lean or non-obese subjects in the absence of other metabolic risk factors[26]. Lean NAFLD is most commonly seen in the Asian population where the parameters for defining obesity are different than those of western population. Non-alcoholic steatohepatitis (NASH) is the severe manifestation since it causes steatosis, inflammation, ballooning and fibrosis which can progress to cirrhosis and hepatocellular carcinoma (HCC). The prevalence of NASH is estimated at 2%-3%[24].

The pathophysiological relationship between DM and NAFLD is bidirectional and complex. On the one hand, T2DM has been suggested as a strong risk factor for NAFLD, LC and HCC[17,18]. In a recently published study with 561 patients with T2DM attending primary care outpatient clinics and



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Figure 1 Pathophysiology of hepatogenous diabetes in cirrhotic liver. Hepatogenous diabetes develops directly from insulin resistance in the liver, and indirectly from impaired glucose metabolism due to insulin resistance in muscle. Hyperinsulinemia can result from reduced insulin clearance by the damaged liver and the presence of portosystemic shunts. With progression of diabetes, there is a reduction in the sensitivity of pancreatic b cells due to glucotoxicity, and reduced production of insulin. IGF: Insulin-like growth factor.

unaware of having NAFLD, 15% showed moderate-to advanced fibrosis by transient elastography and confirmed with liver biopsy. Only a minority of patients showed elevated aspartate aminotransaminase or alanine aminotransaminase[27].

On the other hand, NAFLD in the absence of metabolic disorders may be a risk factor for incidental DM and MS, as it has been demonstrated in lean subjects with NAFLD[28,29]. However, the two conditions have additional common risk factors (Figure 2).

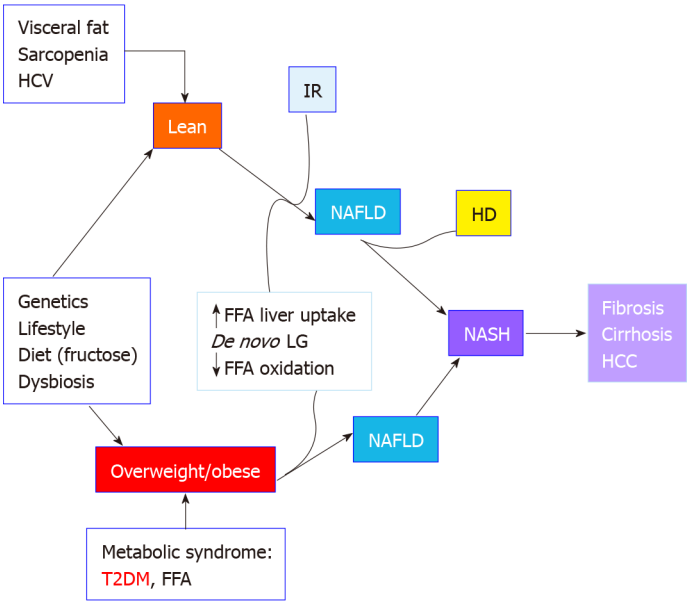
DM in lean patients with NAFLD has clinical characteristics similar to HD. Compared with obese NAFLD patients, leans or non-obese tend to be younger, have lower levels of fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), blood pressure, and homeostasis model assessment IR index (HOMA-IR), and lower prevalence of MS[30]. However, compared with healthy individuals, they tend to have more dyslipidemia and higher FPG, body mass index (BMI), visceral fat, blood pressure and HOMA-IR[31].

HCV AND DM

Numerous prospective studies have shown higher prevalence of DM in patients with chronic hepatitis C virus (CHC) compared to those with HBV liver infection or without liver disease[8,32]. A meta-analysis of 32 studies found that DM was associated with CHC regardless of the presence of fibrosis or LC[33]. The prevalence of DM in patients without LC was 12.6% to 17% and that of IGT was 40%[20,34].

The HCV has diabetogenic properties through several mechanisms. First, autoimmune phenomena, as massive stimulation of the immune system induced by HCV may result in the nonspecific activation of potentially self-reactive lymphocytes that might develop autoimmunity, inducing an immune cascade that could culminate in islet cell dysfunction in susceptible individuals[35]. As a consequence, organ nonspecific antibodies are more frequent in HCV-positive patients with mixed cryoglobulinemia and diabetes than in non-diabetic HCV-negative patients with mixed cryoglobulinemia[36]. Second, direct cytotoxicity to islet β-cells, as rough endoplasmic reticulum morphological changes have been observed in the β-cells of HCV-infected patients, accompanied by reduced glucose-stimulated insulin release[35]. Third, blockade of insulin receptors at the cellular level, as HCV core up-regulates suppressor of cytokine signaling 3 expression that induces proteasomal degradation of insulin receptor substrates 1 and 2 (which are central molecules of the insulin-signaling cascade) and increases gluconeogenesis[37].

The CHC patients with DM have been shown to have an attenuated DM phenotype: They are thinner and have lower levels of low-density lipoprotein (LDL) cholesterol, which could be due to hypobetalipoproteinemia as a result of binding competition between HCV and hepatic LDL receptor, giving rise to



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Figure 2 The pathophysiological relationship between diabetes mellitus and nonalcoholic fatty liver disease. This is bidirectional: On the one hand, type 2 diabetes mellitus (T2DM) is a strong risk factor (alone or as part of metabolic syndrome) for nonalcoholic fatty liver disease (NAFLD), liver cirrhosis and hepatocellular carcinoma. On the other hand, NAFLD in the absence of metabolic disorders is a risk factor for incidental DM as it has been demonstrated in lean subjects with NAFLD. In both cases genetics, [PNPLA3 rs738409 polymorphism (G allele), SREBF-2 rs133291 C/T polymorphism, TM6SF2 rs58542926 C>T and CETP rs12447924 and rs1259700 polymorfisms], as well as sedentary life style, diet and dysbiosis may also play an important role. HCV: Hepatitis C virus; IR: Insulin resistance; NAFLD: Nonalcoholic fatty liver disease; HD: Hepatogenous diabetes; FFA: Free fatty acids; LG: Lactoglobulin; T2DM: Type 2 diabetes mellitus; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

steatosis[38] which is frequently observed in this disease.

Numerous studies have shown that DM has negative clinical implications for the outcomes of HCV liver infection with or without LC[39]. It has been demonstrated that regardless of BMI and age, DM can accelerate the progression of CHC to LC and HCC. In patients with compensated cirrhosis, DM induces an increased risk of decom-pensation with the onset of liver failure and significant increase in mortality [11,40,41]. DM was also associated with an increased risk for HCC development in treatment-naïve CHC patients in Asia. Furthermore, LC and an early DM diagnosis further increased the risk of HCC development in patients diagnosed with both CHC and DM.

With the introduction of direct-acting antiviral (DAA) drugs for the treatment of HCV liver infection, the eradication rate is close to 100% regardless of viral genotype and degree of fibrosis. In multiple studies, the elimination of the virus had a short- and medium-term beneficial effect on DM in CHC patients. In these studies, patients showed improved blood glucose and insulin levels, insulin sensitivity and HbA1c values. These changes were independent of BMI, age and degree of fibrosis[42,43].

In one of these studies with 893 CHC patients, 15.7% with LC, the persistence of normalization of glucose metabolism parameters was demonstrated 44.5 mo after virus eradication[43]. In a recent systematic review and metanalysis HCV eradication with DAAs produced a significant mean reduction in HbA1c levels of 0.45% [95% confidence interval (CI): 0.60-0.30%; $P < 0.001$] and in FPG levels of 22.03 mg/dL (95%CI: 41.61-2.44 mg/dL; $P = 0.03$)[44]. Nevertheless, not all studies have obtained similar results, this may be due to differences in study design, sample size and time of follow up[45]. For this reason, large prospective cohort studies using appropriate stratifications are urgently needed to evaluate the extent of such an amelioration.

CLINICAL MANIFESTATIONS OF DM IN LC, HD

Diagnosis of HD may be difficult since clinical manifestations in the early stages of liver disease are absent. FPG and HbA1c may be normal and in most patients, an oral glucose tolerance test (OGTT) is required for diagnosis[46]. As liver failure progresses, DM becomes clinically manifested[6]. Some clinical parameters may be useful for distinguishing between T2DM and HD. The time of onset is important, as T2DM usually precedes while HD follows the occurrence of LC. Nevertheless, this distinction may be difficult when the two conditions are simultaneously detected. In this case, presence of MS, family history of DM and vascular complications are less frequent, whereas liver-related complications are more frequent in HD compared to T2DM[4,47,48].

Finally, unlike T2DM, orthotopic liver transplantation (LT) may reverse or improve HD confirming its origin from liver dysfunction[49] (Table 1).

Diagnosis

In a study comparing patients with HD to those with T2DM, the ratios of postprandial plasma glucose to FPG, fasting insulin and HOMA-IR index were significantly higher in patients with HD[50]. Consequently, the diagnosis of HD often requires an OGTT. It has been determined that around 50% to 70% of patients with compensated LC will require this test in order to diagnose IGT or DM[4,6,48]. In addition, research is being made to identify genetic and biochemical markers aimed to establish the differentiation among the two types of DM more precisely[50,51].

OGTT

In 1997, the American Diabetes Association determined the cut-off value of FPG in 126 mg/dL for the diagnosis of DM because it corresponded to a 2 h value of 200 mg/dL in the OGTT[52]. A study in 60 LC patients based on the results of OGTT, however, showed that mean values of FPG levels corresponding to a 2 h value of 200 mg/dL were lower (107 mg/dL)[53]. Besides, nine of 42 patients (21%) with FPG levels < 110 mg/dL from this study, were diagnosed with DM using OGTT.

Therefore, lower FPG levels may be required in LC patients for predicting IGT or DM, so the use of lower cut-off values to diagnose HD should be considered in these patients.

HbA1c

HbA1c levels > 6.5%, are used to diagnose DM[54]. However, a previous study showed that LC patients with DM had lower HbA1c levels (mean 5.7%), and 40% of patients with compensated LC had levels below in the non-DM range[55]. The poor diagnostic performance of HbA1c in LC patients is due to the curvilinear relationship between HbA1c and erythrocyte turnover, which can occur in patients with advanced LC as a result of hemorrhage related to portal hypertension and coagulopathy, hemolysis caused by splenomegaly and impaired erythropoiesis due to bone marrow suppression[56]. HbA1c values can also be affected by blood transfusion, which are frequently prescribed to LC patients[57]. A study showed that in non-anemic CLD patients with DM with HbA1c < 7%, the decrease in liver functional reserve is associated with worsening of parameters of glycemic variability determined with continuous glucose monitoring. Mean blood glucose levels and the difference between highest and lowest blood glucose increased significantly with worsening of liver functional reserve[58].

IMPLICATIONS OF DM IN LC

T2DM or HD are associated with numerous complications and high mortality in patients with LC (Tables 2 and 3).

Complications

Hepatic encephalopathy: Several studies have linked DM to an increased incidence of hepatic encephalopathy (HE) in patients with LC. Among patients with HCV-related LC, the severity of HE was higher in DM than in non-DM patients[59]. In a further study in LC patients, the association between DM and HE was independent of the model for end-stage liver disease (MELD) score[39]. In a large prospective study, LC patients with DM had more episodes of first-time overt HE compared to those without DM in one year. In addition, a greater proportion of first-time HE progressed beyond grade 2 in DM patients. Notably, the proportion of Child-Pugh class C LC was lower in the DM group, which suggested that DM conferred an additional risk of HE irrespective of liver disease severity[60].

In a recent study, the risk of both covert and overt HE was more pronounced among patients with poor glycaemic control[61]. Finally, DM increased significantly the risk of HE after a trans-jugular intrahepatic portosystemic shunt (TIPSS)[62].

Variceal haemorrhage: Hyperglycaemia may lead to splanchnic hyperaemia and increased portal pressure which may increase the risks of haemorrhage[59,63]. In a prospective study, DM was associated with increased hepatic venous pressure gradient, variceal haemorrhage (VH), and Child-Pugh's score. Postprandial hyper-glycaemia had a significant association with VH within 6 mo[64]. In another study, DM was a risk factor for rebleeding following endoscopic variceal ligation[65]. In a retrospective study, DM was also an independent predictor of in-hospital death in LC patients with acute gastro-intestinal bleeding[66]. Finally, in another study, LC patients with DM had a higher incidence of re-bleeding and hospitalizations, and a higher mortality rate than those without DM[67].

Infectious complications

An impaired immunological response has been observed in patients with DM and LC[68]. DM was an independent predictor of bacterial infections in hospitalized patients with LC[40]. In hospitalized LC patients, the prevalence of bacterial infections was significantly higher in DM compared to non-DM

Table 1 Clinical differences between hepatogenous diabetes and type 2 diabetes mellitus

Variables	Hepatogenous diabetes	Type 2 diabetes mellitus
Onset	After cirrhosis onset	Before cirrhosis onset
Clinical presentation	Normal FPG and HbA1c; Abnormal OGTT	Increased FPG and HbA1c
Metabolic risk Factors	Less frequent	More frequent
Vascular complications	Less frequent	More frequent
Liver complication	More frequent	Less frequent
Effect of OLT	Reversal or improvement	Non modification
Mortality	More than non-diabetics	More than non-diabetics

FPG: Fasting plasma glucose; OGTT: Oral glucose tolerance test; OLT: Orthotopic liver transplantation; HbA1c: Glycated hemoglobin.

Table 2 Studies depicting implications of diabetes on complications of patients with liver cirrhosis

Ref.	Design	Population, *n*	Outcomes	Limitations
Sigal *et al*[59], United States, 2006	Cross-sectional	65 HCV-LC; 31% diabetics	HE and severe HE was higher in diabetics. DM was independent risk factor for HE	Small sample size. HE was not standardized
Tietge *et al*[81], Germany, 2004	Case-control, prospective	100 LC, 35% diabetics, 62 post-LT	Pre-LT IGT or DM was the major risk factor for post-LT DM	Only 31 patients were prospectively evaluated
Takahashi *et al*[77], Japan, 2011	Prospective	203 CHC	Two hours post-challenge hyperglycaemia associated with HCC	Patients received IFN
Jeon *et al*[64], Republic of Korea, 2013	Prospective	195 LC, 55.4% with HD	HD correlated with HVPG, VH and large varices. Most patients with VH within 6 mo, had post-prandial hyperglycaemia	Risk stratification of varices and prophylaxis for VH were not taken into account
Zheng *et al*[75], China, 2013	Retrospective case-control	1568 CLD, 852 with HCC	DM associated with increased risk of HCC regardless of cirrhosis. Synergistic interaction between DM and HBV for HCC	Hospital based study. Temporal relationship between DM and HCC could not be established
Yang *et al*[63], Taiwan, 2014	Prospective	146 LC, 25% diabetics	DM was predictor of VH. Patients with VH had worse glycaemic control (HBA1c ≥ 7%)	DM associated with decompensated cirrhosis, renal disease and VH
Jepsen *et al*[60], Denmark, 2015	Database from randomized trials	863 LC, 22% diabetics	Diabetics had more episodes of first-time overt HE in one year. First-time HE progression beyond grade 2 higher in diabetics	Diagnosis of DM was not standardized. Vaptan could be a confounder
Yang *et al*[73], United States, 2016	Retrospective	739 LC, 34% diabetics	DM increased the risk of HCC in patients with non-HCV cirrhosis	Single-centre probably with referral bias
Tergast *et al*[69], Germany, 2018	Prospective case-control	475 decompensated LC, 118 diabetics	DM increased risk for SBP and was higher with HbA1c values ≥ 6.4%	Criteria for diagnosis of DM not clearly defined
Wang *et al*[65], China, 2020	Retrospective	207 LC, 137 diabetics; 68 had HD	Rebleeding rate following EST or EVL higher in diabetics, including HD at 1, 3, and 6 mo	Relatively small number of patients with shorter follow-up
Labenz *et al*[61], Germany, 2020	Prospective	240 LC, 27% diabetics	DM associated with covert HE at inclusion and follow-up. The risk of covert HE and overt HE was more pronounced when HbA1c ≥ 6.5%	Spontaneous porto-systemic shunts, GIB, drugs were not taken into account

DM: Diabetes mellitus; EST: Endoscopic sclerotherapy; EVL: Endoscopic variceal ligation; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HD: Hepatogenous diabetes; HE: Hepatic encephalopathy; IGT: Impaired glucose tolerance; OGTT: Oral glucose tolerance test; VH: Variceal hemorrhage; LT: Liver transplantation; HVPG: Hepatic venous pressure gradient; HBV: Hepatitis B virus; IFN: Interferon; GIB: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis; LC: Liver cirrhosis; HbA1c: Glycated hemoglobin.

subjects[68]. In a prospective study in LC patients with ascites, those with DM had an increased risk of developing spontaneous bacterial peritonitis (SBP) and the incidence of SBP was significantly higher when HbA1c values were ≥ 6.4%[69]. In a retrospective analysis of LC patients with DM, uncontrolled DM was associated with an increased overall risk of bacterial infection and a higher hospital mortality rate in the elderly[70].

Table 3 Prospective and retrospective studies depicting implications of diabetes on mortality of patients with liver cirrhosis

Ref.	Design	Population	Outcomes	Limitations
Bianchi *et al*[3], Italy, 1994	Retro-prospective	354 LC, 98 with DM	5-yr survival rate: DM: 41%, non-DM 56%	Diagnosis of DM not standardized
Holstein *et al*[4], Germany, 2002	Prospective	52 LC, 71% with DM	5.6-yr survival rate after diagnosis of LC: 51% of HD patients. 80% of deaths were cirrhosis-related causes	Small sample size. Comparative outcome data of non-DM patients not available
Moreau *et al*[79], France, 2004	Prospective	75 LC and refractory ascites	DM, older age, and HCC were predictors of poor survival. The survival rate of patients without DM was higher	OGTT was not used to diagnose DM
Nishida *et al*[48], Japan, 2006	Prospective	56 LC, 38% diabetics	The 5-yr survival rate was 94%, 68% and 56%, with NGT, IGT and DM, respectively	Small sample size
Quintana *et al*[80], México, 2011	Prospective	110 compensated LC, 45% diabetics	2.5 yr cumulated survival years: DM: 48 *vs* non-DM: 69% ($P < 0.05$). DM was not predictor of death	Maybe DM death- prediction capability was masked by Child-Pugh C score
García-Compeán *et al*[78], México, 2014	Prospective	100 compensated LC and normal FPG	Patients with IGT + DM had lower 5-yr cumulated survival rate. Death causes in 90 % were cirrhosis related	Small sample size
Elkrief *et al*[40], Canada, 2014	Retrospective	348 HCV-LC, 40% diabetics	DM significantly associated with ascites, renal dysfunction, infections, HCC and mortality during the follow-up period	Retrospective. Potential errors in the diagnosis of DM
Khafaga *et al*[67], Egypt, 2015	Case-control	60 LC, 50% diabetics	Diabetics had higher incidence of VH, hospitalizations, HE and mortality rate	Small sample size
Qi *et al*[66], China, 2015	Retrospective	145 LC, 29 diabetics	In-hospital mortality was higher in diabetics	Small number of patients
Hoehn RS *et al*[82], United States, 2015	Retrospective	12442 pos- LT, 24% with DM	Diabetic recipients had longer hospitalization, higher peri-transplant mortality and 30-d readmission rates	More diabetic patients were on haemodialysis and received allografts from older donors
Rosenblatt *et al*[70], United States, 2021	Retrospective	906559 LC with DM, and 109694 uncontrolled DM	Uncontrolled DM associated with increased risk of bacterial infection and increased risk of death in elderly patients	Subject to administrative error. Criteria for DM was not standardized

DM: Diabetes mellitus; FPG: Fasting plasma glucose; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HD: Hepatogenous diabetes; HE: Hepatic encephalopathy; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; OGTT: Oral glucose tolerance test; VH: Variceal hemorrhage; LT: Liver transplantation; LC: Liver cirrhosis.

HCC

The increased risk of HCC in diabetic patients was reported in two large meta-analyses[71,72]. DM was also found to increase the risk of mortality in HCC patients[71]. However, the published studies are somewhat inconsistent on this issue. In a retrospective study with LC patients, DM did not increas the risk of HCC in those with HCV infection compared to other causes of liver disease[73]. Another study in patients with chronic hepatitis B, reported a higher prevalence of DM among HCC patients without LC than in those with LC[74]. A large case control study found that DM was associated with an increased risk of HCC regardless of the prevalence of LC[75]. In another study, DM could not be confirmed as a major risk factor for HCC in general; however, DM did become an independent predictor when "traditional" risk factors such as LC, alcohol abuse, hepatitis B or C and smoking were excluded[76]. The inconsistent criteria for diagnosing DM may be an important cause of this discrepancy. Recently, it was observed that while high FPG levels were not associated with HCC, 2 h post-challenge hyperglycaemia remained as significant predictor for HCC development in HCV-RNA-positive patients [77].

Mortality

Many studies have indicated that DM significantly reduced the survival rate in patients with CLD and LC[3,4,40,48,66,67,78,79]. Nevertheless, only some of them have been prospective. Bianchi *et al*[3] reported the adverse impact of DM on the 5-year cumulated survival in a study in patients with LC. Refractory ascites, DM, older age, and HCC, but not Child-Pugh score were independent predictors of poor survival[3]. Even subclinical abnormalities in glucose homeostasis have been found to adversely affect prognosis. In a recent prospective study of 100 compensated LC patients with normal FPG, those with abnormal OGTT had lower 5-year cumulated survival than those with a normal test[78]. In a similar prospective study, the cumulative 5-year survival rates of patients were 94.7%, 68.8% and 56.6%, in those with normal glucose tolerance, IGT and DM, respectively[48]. In another study, DM had an

impact on survival only in patients with a baseline MELD score < 10. This implies that the severity of CLD may mask the deleterious effect of DM[40,80]. The results of these studies suggest that DM can be detected more clearly as reliable predictor of morbidity and mortality in the early stages of LC. In the advanced stages, its effect can be masked by other complications of LC. Therefore, further studies, based on the dynamic assessment of glycaemic parameters using OGTT, are needed to obtain a robust conclusion on this important issue (Table 3).

LT

Pre-transplant DM is the major risk factor for DM after LT (7%-45%)[81-83]. Increased FPG levels were a risk factor for new-onset DM after LT[84]. Pre-operative β-cell function determined by an OGTT may be a useful predictive tool for the recurrence of DM after LT[85]. Post-LT DM is associated with increased risk of graft rejection, severe complications and mortality[81,86,87]. A study on adult LT recipients showed that post-LT DM incidence was 34.7%, 46.9%, and 56.2% at 1, 3, and 5 years, respectively, with overall survival rates of 90%, 80.9%, and 71.7%, respectively. The post-LT DM group had more rejection episodes and worse 5-year survival rates[86]. Persistent or new-onset DM after LT is also associated with cardiovascular disease, biliary complications, renal dysfunction, infections and graft rejection[81, 87]. In patients with HCV-related LC, a pre-existing or new onset DM is associated with increased risk of HCV recurrence and hepatic fibrosis after LT[88]. Some studies have demonstrated improvement in glucose homeostasis after LT[49,89]. In a study where LT failed to cure overt DM in 33% of patients, a persistently reduced β-cell function was found[88]. However, normalized glucose production and insulin sensitivity after LT have the potential to reverse β-cell dysfunction and thus lead to remission in most cases of HD[90].

MANAGEMENT OF DM IN LC

In the absence of specific guidelines, the treatment of DM in patients with LC (T2DM or HD) starts from the general principles of management of T2DM, according to current established guidelines[91].

Lifestyle

Diet and physical activity are a cornerstone of T2DM management. On the one hand, prevalence of obesity and NAFLD is increasing worldwide and > 10% of weight reduction has been shown to significantly reduce inflammation and fibrosis in patients with this disease[92]. On the other hand, malnutrition remains a common feature among LC individuals (20%-50%), mostly in those with decompensated liver disease[93]. Both obesity and malnutrition may be associated with sarcopenia, causing a major risk factor for frailty, conditions associated with a higher rate of severe complications [94].

Diet

A moderate caloric restriction is recommended for overweight/obese LC patients in order to achieve a weight reduction of > 5% to 10%, but paying a special attention to maintain an adequate protein intake to avoid loss of muscle mass (85%). A widely accepted approach is to supply at least 35 kcal/kg body weight/d, using the actual body weight, then subtracting a 5%, 10%, or 15% in case of mild, moderate, or severe ascites, respectively, plus an additional 5% in case of peripheral oedema[93].

Protein intake should be increased up to 1.2-1.5 g/kg body weight/d to avoid sarcopenia, unless moderate-to-severe renal insufficiency is present, but oral protein supplements, especially branched-chain amino acids[95], or short-term enteral or parenteral nutrition[93] may be necessary in some patients. The common deficits of vitamins should be corrected with supplementation[93].

Physical activity

Physical exercise is associated with increments of insulin sensitivity and is highly recommended in patients with NAFLD. However it may be limited in LC patients by the presence of asthenia, sarcopenia, and ascites[96,97]. A combination of aerobic and resistance training of moderate intensity is also recommended[93], as it may result in the concurrent improvement of muscle function and mass.

Pharmacological therapy

Despite the growing problem of management of DM in patients with CLD the existing literature data, especially on newer antidiabetic agents is very limited and furthermore, no guidelines exist. The recommended use of antidiabetic drugs and insulins is based mostly on available data on pharmacokinetics and safety drug studies taking into account the degree of liver dysfunction and the presence of comorbidities[98] (Tables 4 and 5).

Non-insulin agents

The inhibitors of alpha-glucosidase such as the acarbose, inhibit α-glucosidases, which contribute to

Table 4 Kinetics, metabolism and excretion of the currently available anti-hyperglycaemic drugs[102]

Drug	Half life	Metabolism	Excretion
Short-acting insulins			
Human	140 min	Proteolytic degradation	
Lyspro	80 min	Proteolytic degradation	
Aspart	80 min	Proteolytic degradation	
Glulisine	80 min	Proteolytic degradation	
Long-acting insulins			
Human-NPH	6.6 h	Proteolytic degradation	
Glargine	12.1 h	Proteolytic degradation	
Levemir	5-7 h	Proteolytic degradation	
Degludec	25 h	Proteolytic degradation	
Glargine-300	19 h	Proteolytic degradation	
Sulfonylureas			
Glibenclamide	10 h	Liver 100%	Urines 50%; feces 50%[1]
Glimepiride	9 h	Liver 100%	Urines 60%; feces 40%[1]
Gliclazide	10-11 h	Liver 100%	Urines 80%; feces 20%
Glipizide	2-5 h	Liver 90%	Urines mainly
Meglitinides			
Repaglinide	1 h	Liver 100%	Bile 92%; urines 8%
Biguanides			
Metformin	1.5-3 h	Not metabolised	Urines 100%
Thiazolidinediones			
Pioglitazone	3.7 h	Liver 100%	Feces 55%; urines 45%
DPP-4 inhibitors			
Sitagliptin	8–24 h	Limited	Urines
Vildagliptin	1.5–4.5 h	Limited	Urines
Saxagliptin	2–4 h	Moderate	Urines
Linagliptin	10–40 h	Extensive	Feces
Alogliptin	12–21 h	Limited	Urines
GLP-1RAs			
Exenatide	2.4 h	Proteolytic degradation	Renal
Liraglutide	13 h	Proteolytic degradation	No specific organ
Lixisenatide	3 h	Proteolytic degradation	Renal
Exenatide LAR	5-6 d	Proteolytic degradation	Renal
Dulaglutide	5 d	Proteolytic degradation	No specific organ
Semaglutide	7 d	Proteolytic degradation	No specific organ
α-glicosidase inhibitors			
Acarbose	4 h	Intestine	Urines 35%; feces 65%
SGLT2 inhibitors			
Dapaglifozin	10-13 h	Glucuronidation	Urines 33%; feces 42%
Canaglifozin	12.9 h	Glucuronidation	Urines 75%; feces 21%
Empaglifozin	12.4 h	Glucuronidation	Urines 54%; feces 41%

Ertugliflozin	17 h	Glucuronidation	Urines 50%; feces 41%

[1]Excreted as weakly active metabolite.

DPP-4: Dipeptidyl peptidase 4; GLP-1RAs: Glucagon-like peptide 1 receptor agonists; SGLT2: Sodium-glucose cotransporter 2; LAR: Long-acting release; NPH: Neutral protamine Hagedorn.

degradation of disaccharides in the intestine. It results in reduction in the absorption of carbohydrates and in the risk of postprandial hyperglycemia. Its safety has been evaluated in patients with DM and CLD[99,100]. Its use was associated with a significant reduction of fasting and postprandial hyperglycemia, HbA1c and C-peptide as well as improvement of mild HE in compensated cirrhosis [100].

The secretagogues sulfonylureas and glinides are extensively metabolized by the liver in a cytochrome P450-dependent manner and may accumulate in LC patients[99], thus increasing the risk of hypoglycemia[101]. Thus, it is recommended to avoid these agents in patients with moderate-to-severe liver failure.

The insulin-sensitizing agent metformin is not metabolized by the liver[102]. It has been associated with risk of lactic acidosis. However, this complication was reported only in anecdotal cases, particularly with concomitant alcohol intake[103]. Chronic use of metformin has been associated with a reduced risk of HCC[104-107], reduced liver-related complications[105,107], and increase survival in LC patients[105-107].

Probably, glycemic control of the patients contributed for obtaining these effects. Nevertheless, an independent anti oncogenetic mechanism has been recently described experimentally[108]. The other insulin-sensitizing agents, thiazolidinediones, are metabolized entirely by the liver, so they accumulate in patients with hepatic failure[101]. Therefore, their use is restricted to patients with Child-Pugh class A LC, also because of the fluid retention and decrease in bone mineral density caused by these drugs[109]. The significant reduction of liver fibrosis reported in NASH patients with and without T2DM supports the use of these drugs in early-stage LC due to this aetiology.

Despite the fact that all the inhibitors of dipeptidyl peptidase 4 (DPP-4) are metabolized by the liver, their use is generally allowed in patients with Child-Pugh class A or B LC with no dose adjustment[101,110]. A dose reduction of these drugs is however required in case of estimated glomerular filtration rate (eGFR) < 50 mL · min^{-1} · 1.73m^{-2}, except for linagliptin, which is not excreted by the kidney[111]. Notwithstanding, in a recently published population based cohort study with 2828 DPP-4 inhibitor user and nonuser patients with T2DM and LC, the incidence rate of decompensated cirrhosis during follow-up was significantly higher for DPP-4 inhibitor users. The adjusted hazard ratios (aHRs) (95%CI) of variceal bleeding and hepatic failure were 1.67 (1.11-2.52) and 1.35 (1.02-1.79), respectively, for DPP-4 inhibitor users over nonusers. The risk of all-cause mortality, HCC, and major cardiovascular events were not statistically different[112]. In another population-based, retrospective cohort study including patients with DM and LC treated with GLP-1 receptor agonists (GLP-1RAs), DPP-4 inhibitors, sulfonylureas or sodium-glucose co-transporter-2 (SGLT-2) inhibitors, GLP-1RAs use was associated to significantly reduced number of liver related complications compared to DPP-4 inhibitors and sulfonylureas. In contrast, complication rates were similar when GLP-1RAs and SGLT-2 inhibitors were directly compared[113]. The results of these studies suggest that patients using DPP-4 inhibitors should heave a tight monitoring.

The GLP-1RAs, are not metabolized by the liver and, hence, no dose adjustment is required[101,110]. As pharmacokinetic information in patients with end-stage liver disease are available only for liraglutide, dulaglutide, and semaglutide, so the use of these GLP-1RAs is allowed in Child-Pugh class A or B patients, whereas that of exenatide and lixisenatide should be restricted to Child-Pugh class A individuals[101,110]. The use of exenatide and lixisenatide should be avoided also if eGFR is < 30 mL · min^{-1} · 1.73 m^{-2}, as these agents are excreted by the kidney[111]. The reported beneficial impact of this class of drugs on NASH may support their use in patients with NASH-related LC. Conversely, these agents may not be suitable for malnourished sarcopenic individuals, due to their energy wasting effect [101].

The inhibitors of the SGLT-2 are all metabolized in the liver, but have significant accumulation only in severe liver failure[114]. Therefore, the use of SGLT2 inhibitors is allowed in Child-Pugh class A and B LC with no dose adjustment[101], unless impaired renal function is present[111]. Their diuretic properties might be useful for potentiating the effect of therapy with loop diuretics and mineralocorticoid receptor agonists[115], though they may cause dehydration that may further reduce effective plasma volume and precipitate renal dysfunction[101].

In general terms, the use of non-insulin agents (except secretagogues) is generally allowed in mild-to-moderate LC, whereas all of them should be avoided in severe LC, in which insulin represents the sole treatment option[101]. In HD, metformin alone may be sufficient. However, drugs potentially capable of preserving β-cell function (*e.g.*, thiazolidinediones, incretin-based drugs, and SGLT2 inhibitors) may be also suggested[101].

Table 5 Use of anti-hyperglycaemic agents in cirrhotic individuals according to Child-Pugh class[102]

Drug	Child-Pugh class A	Child-Pugh class B	Child-Pugh class C
Short-acting insulins			
Human	Allowed	Allowed	Allowed (dose reduction)
Lyspro	Allowed	Allowed	Allowed
Aspart	Allowed	Allowed	Allowed
Glulisine	Allowed	Allowed	Allowed
Long-acting insulins			
Human-NPH	Allowed	Allowed	Allowed (dose reduction)
Glargine	Allowed	Allowed	Allowed
Levemir	Allowed	Allowed	Allowed
Degludec	Allowed	Allowed	Allowed
Glargine-300	Allowed	Allowed	Allowed
Sulfonylureas			
Glibenclamide	Not recommended	Contraindicated	Contraindicated
Glimepiride	Allowed (caution)	Not recommended	Contraindicated
Gliclazide	Allowed (caution)	Not recommended	Contraindicated
Glipizide	Allowed (caution)	Not recommended	Contraindicated
Meglitinides			
Repaglinide	Allowed (caution)	Not recommended	Contraindicated
Biguanides			
Metformin	Allowed	Allowed (dose reduction)	Contraindicated
Thiazolidinediones			
Pioglitazone	Allowed	Contraindicated	Contraindicated
DPP-4 inhibitors			
Sitagliptin	Allowed	Allowed	Contraindicated
Vildagliptin	Contraindicated	Contraindicated	Contraindicated
Saxagliptin	Allowed	Allowed	Contraindicated
Linagliptin	Allowed	Allowed	Contraindicated
Alogliptin	Allowed	Allowed	Contraindicated
GLP-1RAs			
Exenatide	Allowed	Contraindicated	Contraindicated
Liraglutide	Allowed	Contraindicated	Contraindicated
Lixisenatide	Allowed	Allowed	Contraindicated
Exenatide LAR	Allowed	Allowed	Contraindicated
Dulaglutide	Allowed	Allowed	Contraindicated
Semaglutide	Allowed	Allowed	Contraindicated
α-glicosidase inhibitors			
Acarbose	Allowed	Allowed (caution)	Contraindicated
SGLT2 inhibitors			
Dapaglifozin	Allowed	Allowed	Contraindicated
Canaglifozin	Allowed	Allowed	Contraindicated
Empaglifozin	Allowed	Allowed	Contraindicated

Ertugliflozin	Allowed	Allowed	Contraindicated

DPP-4: Dipeptidyl peptidase 4; GLP-1 RAs: Glucagon-like peptide 1 receptor agonists; SGLT2: Sodium-glucose cotransporter 2; NPH: Neutral protamine Hagedorn; LAR: Long-acting release.

Insulin

As human insulin is metabolized by insulinase in the liver, it may be necessary to reduce dosage[116]. Conversely, as no significant changes in the kinetics of insulin analogues, either both short-acting[117], or long-acting insulins[101,116] have been reported, no dose adjustment is required for these agents.

For these reasons, use of insulin (with preference for insulin analogues) is allowed at all stages of cirrhosis (Table 5) and represents the first-choice treatment in LC patients with DM. Insulin requirements can be high in patients with compensated cirrhosis and low in decompensated patients. Few clinical studies have evaluated its long-term effects and safety. In a recently published retrospective cohort study, insulin use in people with T2DM and compensated LC was associated with higher risks of hypoglycemia, cardiovascular events, liver-related complications, and mortality than insulin nonusers. However, no information regarding important risk factors such as body weight, physical activity, alcohol consumption, and cigarette smoking was given and effective glycemic control or treatment adherence was not evaluated[118]. More studies are needed to confirm these findings. Anyway, insulin treatment should be started with close monitoring to avoid hypoglycemia. The insulin regimen may consist of basal insulin only or a combination of basal and prandial insulin (basal-plus or basal-bolus).

In the Figure 3 an algorithm for the diagnosis and management of DM in CLD and LC is depicted.

Treatment of post-transplantation DM

HD may be improved with LT, nevertheless it may persist in 30% of cases or DM may occur *de novo* after LT due to several factors with diabetogenic potential, such as immunosuppressant treatment, viral infections, and donor- and procedure-related factors[101].

The intra-operative and immediate post-LT periods are often characterized by severe hyperglycemia that may be transient and reverse with appropriate management[101]. Intravenous or subcutaneous intensive insulin therapy using validated algorithms is the standard of care, as a strict intra-operative glycemic control is recommended to reduce the associated increased risk of morbidity and mortality [119]. With reduction of steroid dose, insulin requirement rapidly decreases and insulin treatment may be interrupted in many instances[101].

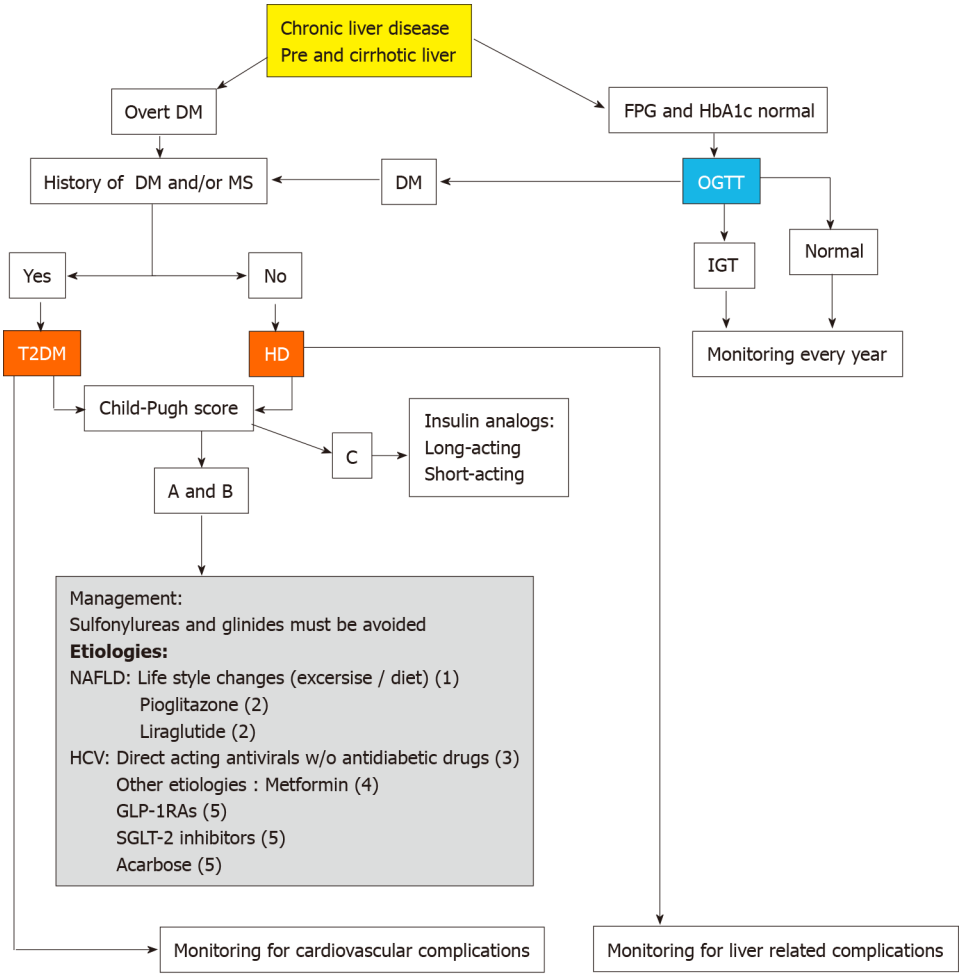
Specific guidelines for the treatment of post-LT DM were released in 2014[83]. While lifestyle measures are identical to those for T2DM patients[91], there are insufficient data to recommend specific anti-hyperglycemic agents[101]. Indeed, all the available agents can be used to treat post-transplant DM [120], with limitations in case of renal dysfunction[121].

CONCLUSION

DM and IGT are common in LC patients worldwide, particularly in this current era of NAFLD and obesity. The pathophysiological relationship of DM and LC is bidirectional. Over the years, the evidences that LC is a diabetogenic condition have been consolidated and the mechanisms are better understood. High-quality evidences have also determined that DM is associated to increased complications and death in CLD patients. Although the existence of two types of DM in LC patients has been confirmed, the practical usefulness of taxonomic separation of the two types of DM is unknown. In part because they have not been separately studied.

However, the research carried out to date has permitted to clearly understand the pathophysiologic mechanisms of HD and to define its clinical characteristics. Despite this, HD is not currently recognized as secondary diabetes nor as a complication of LC.

Based on the arguments presented in this review, we think that, it is time to classify LC-associated DM into T2DM and HD in order to standardize clinical research studies, which will make it possible to evaluate separately their impact on outcomes of LC patients. It is also urgent to determine standardized therapeutic guidelines for this vulnerable patients based on prospective randomized clinical trials with great number of patients and long term follow up taking into account clinical surrogates such as complication and mortality rates. Because these patients are referred from primary care levels to specialized services, we believe that the hepatologists should have the basic knowledge in the management of uncomplicated DM and equally the diabetologists should have the basic competences in the early detection and management of CLD. Complicated and severe patients should be treated by a multidisciplinary team.



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Figure 3 Algorithm for diagnosis and management of diabetes mellitus and nonalcoholic fatty liver disease based on the published evidences. As follows: (1) This treatment has been evaluated only in nonalcoholic fatty liver disease (NAFLD); (2) These drugs have been evaluated in NAFLD showing improvement of non-alcoholic steatohepatitis and fibrosis; (3) Direct-acting antiviral have demonstrated improvement of short and long term glycemic control after hepatitis C virus eradication; (4) Long term administration of metformin has demonstrated association to significant reduction of liver related complications, hepatocellular carcinoma and mortality; and (5) GLP-1 receptor agonists and sodium-glucose co-transporter-2 inhibitor drugs have demonstrated effectiveness for glycemic control and good tolerance in liver cirrhosis patients. NAFLD: Nonalcoholic fatty liver disease; DM: Diabetes mellitus; GLP-1Ras: GLP-1 receptor agonists; SGLT-2: Sodium-glucose co-transporter-2; HCV: Hepatitis C virus; FPG: Fasting plasma glucose; HbA1c: Glycated hemoglobin; HD: Hepatogenous diabetes; MS: Metabolic syndrome; OGTT: Oral glucose tolerance test; IGT: Impaired glucose tolerance.

FOOTNOTES

Author contributions: García-Compeán D, Orsi E, Kumar R, Gundling F and Nishida T made a bibliographic research and wrote sections of the manuscript, reviewed and corrected the final text; Cueto-Aguilera ÁN made a bibliographic research; Villarreal-Pérez JZ, González-González JA and Pugliese G critically reviewed the manuscript; García-Compeán D conceived and coordinated the whole project.

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Country/Territory of origin: Mexico

ORCID number: Diego García-Compeán 0000-0002-9642-8567; Emanuela Orsi 0000-0002-1255-8437; Ramesh Kumar 0000-0001-5136-4865; Felix Gundling 0000-0002-1481-7674; Tsutomu Nishida 0000-0003-4037-9003; Jesús Zacarías Villarreal-Pérez 0000-0002-6814-062X; Ángel N Del Cueto-Aguilera 0000-0002-6506-1977; José A González-González 0000-0003-4858-5717; Giuseppe Pugliese 0000-0003-1574-0397.

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MINIREVIEWS

Mixed neuroendocrine–nonneuroendocrine neoplasms of the gastrointestinal system: An update

Gulsum Ozlem Elpek

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Gulsum Ozlem Elpek, Pathology, Akdeniz University Medical School, Antalya 07070, Turkey

Corresponding author: Gulsum Ozlem Elpek, MD, Professor, Pathology , Akdeniz University Medical School, Dumlupınar bulvarı, Antalya 07070, Turkey. elpek@akdeniz.edu.tr

Abstract

Mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs) of the digestive tract are a rare heterogeneous group of tumors that present many challenges in terms of diagnosis and treatment. Over the years, the diagnostic criteria, classification, and clinical behavior of these tumors have been the subjects of ongoing debate, and the various changes in their nomenclature have strengthened the challenges associated with MiNENs. This review is performed to provide an understanding of the key factors involved in the evolution of the designation of these tumors as MiNEN, highlight the current diagnostic criteria, summarize the latest data on pathogenesis and provide information on available treatments. Moreover, this work seeks to increase the awareness about these rare neoplasms by presenting the clinicopathological features and prognostic factors that play important roles in their behavior and discussing their different regions of origin in the gastrointestinal system (GIS). Currently, the MiNEN category also includes tumors in the GIS with a nonneuroendocrine component and epithelial tumors other than adenocarcinoma, depending on the organ of origin. Diagnosis is based on the presence of both morphological components in more than 30% of the tumor. However, this value needs to be reconfirmed with further studies and may be a limiting factor in the diagnosis of MiNEN by biopsy. Furthermore, available clinicopathological data suggest that the inclusion of amphicrine tumors in the definition of MiNEN is not supportive and warrants further investigation. The diagnosis of these tumors is not solely based on immunohistochemical findings. They are not hybrid tumors and both components can act independently; thus, careful grading of each component separately is required. In addition to parameters such as the metastatic state of the tumor at the time of diagnosis and the feasibility of surgical resection, the aggressive potential of both components has paramount importance in the choice of treatment. Regardless of the organ of origin within the GIS, almost MiNENs are tumors with poor prognosis and are frequently encountered in the elderly and men. They are most frequently reported in the colorectum, where data from molecular studies indicate a monoclonal origin; however, further studies are required to provide additional support for this origin.

Key Words: Mixed neuroendocrine–nonneuroendocrine neoplasms; Mixed adeno neuroendocrine carcinoma; Gastrointestinal system; Liver; Pancreas; Gallbladder

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Core Tip: Mixed neuroendocrine-nonneuroendocrine neoplasms of the gastrointestinal system are a rare heterogeneous group of tumors that present many challenges in terms of diagnosis and treatment. Current data indicate that they are more frequent in the colon and rectum and that most of them consist of aggressive tumors that have poor prognoses in older men. Their correct diagnosis with the proposed criteria and the separate assessment of the grade of each component are crucial in terms of determining the treatment. Although studies have indicated a monoclonal origin, further studies are needed to determine whether these molecular changes could become treatment targets.

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INTRODUCTION

In epithelial tumors, cells with both neuroendocrine and nonneuroendocrine features coexist in varying amounts, and these tumors occur in almost all organs, including those of the GIS. Although these rarely encountered neoplasms are well known in terms of pathology, debates remain about their diagnosis, classification, pathogenesis, behavior and treatment, and some points are still controversial.

The presence of different proportions of each component in a mixed epithelial tumor (each of which can account for 1% to 99% of the tumor) can result in a wide variety of morphologically heterogeneous tumors as well as different classifications and diagnostic difficulties in pathology[1]. More importantly, these conditions have created problems for oncologists in determining the component that should be targeted primarily in treatment. In addition, the assignment of different definitions to mixed epithelial tumors has led to great inconsistencies in the data obtained from previous studies, especially in determining the prognostic parameters that affect their behavior[2,3]. Therefore, different criteria have recently been introduced for simplifying the diagnosis and classification of these tumors based on the identification of prognostic parameters that may enable effective treatment for mixed epithelial tumors.

The purpose of this review is to highlight the definition of mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs), summarize the current approaches for their histopathological diagnosis and molecular findings related to their pathogenesis and address current approaches in their treatment. Moreover, this work is performed to raise awareness about these rare tumors of the GIS by presenting localization-specific demographic and clinicopathological findings.

DEFINITION OF MiNEN

Mixed tumors were first described by Cordier[4] at the beginning of the last century as neoplasia in the gastrointestinal tract consisting of adenocarcinoma and neuroendocrine components. However, their classification as a different group and their subcategories under this heading were not recommended until proposed by Levine in 1967[5]. Accordingly, three main subtypes have been proposed based on their nonneuroendocrine and neuroendocrine features: collision tumors, combined tumors, and amphicrine tumors. However, this nomenclature has not been entirely accepted. In addition to the challenges posed by their pathological heterogeneity, many different definitions (some repetitious or overlapping) have been provided for mixed epithelial neoplasms composed of both neuroendocrine and nonneuroendocrine components, which has led to further confusion among clinicians and pathologists by leading to considerable variability in published data[2]. In recent years, many attempts have been made to elucidate the clinical and biological meanings of the different combinations of components and simplify the diagnosis and classification, with the primary goal of developing precise diagnostic criteria that can be used to produce a proper prognostic classification for the management of patients. Standardizing the terminology to provide a prognostic classification of mixed neoplasms of the digestive tract was first suggested by Capella *et al*[6] in 2000. Accordingly, the term "mixed exocrine–endocrine tumor" was used by the World Health Organization (WHO) to define these neoplasms[7]. This category included previous subcategories suggested by Levine[5], whereas adenocarcinomas or

squamous cell carcinomas with scattered neuroendocrine cells were excluded based on previous data about the absence of a relationship between the presence of neuroendocrine cells and prognosis. To emphasize the diagnosis, a subjective cutoff value of 30% was determined for each component to define mixed tumors. Ten years later, the term "mixed exocrine–endocrine tumor" was substituted by "mixed adeno-neuroendocrine tumor" (MANEC)[8]. Since the two components of mixed tumors of the GIS are not always constituted by adenocarcinomas and neuroendocrine adenocarcinoma (NEC), the term MANEC was not sufficient to describe these combinations. This situation encouraged many researchers to find another term that included other associations. In 2016, La Rosa *et al*[2] proposed the umbrella term "mixed nonneuroendocrine and neuroendocrine neoplasm" (MiNEN). Introduced first for the pancreas by the 2017 WHO classification of tumors of endocrine organs, this term is currently used for all mixed neoplasms of the GIS[9]. According to the WHO, mixed neoplasms consisting of an adenoma and a neuroendocrine tumor (NET) should be classified as MANETs and should not be included in the MiNEN group.

DIAGNOSIS OF MiNEN

Pathological evaluation of H&E-stained sections is essential for detecting the neuroendocrine and nonneuroendocrine components for the diagnosis of MiNEN. These findings must be confirmed by immunohistochemical (IHC) evaluation. IHC markers used in detecting and grading the endocrine component should be accompanied by markers appropriate for the type of nonneuroendocrine component (Figure 1). The properties and applications of these markers for neuroendocrine neoplasia (NEN) will be briefly mentioned here. However, comprehensive information on this subject can be reviewed in a previous study[10]. Although several biomarkers, including neuron-specific enolase (NSE), CD57, protein gene product 9.5 (PGP 9.5), insulinoma-associated protein 1 (INSM1) and somatostatin receptor subtype 2A (SSTR2A), have been described to date, the most widely used and reliable neuroendocrine markers are chromogranin A, synaptophysin, and CD56[11,12]. In the nonneuroendocrine component, adenocarcinomas express carcinoembryonic antigen, CA 19-9, cytokeratins 7, 19, and AE 1/3. The immunohistochemical features of other tumors that make up this component are presented below according to their localization in different organs of the GIS[10-12].

Both tumor components must account for at least 30% of the whole neoplasm for the diagnosis of MiNEN[8]. This cutoff is arbitrary and was proposed in 1987. The basis for determining this value is the assumption that the prognosis is influenced by the predominant histological component and prevents the management of these cases without consideration of treatment guidelines[1,5]. However, this value has not been reaffirmed in systematic studies. Moreover, the possibility of the negative influence of a small component of high-grade NEN on tumor behavior should not be overlooked[13,14]. This quantitative threshold also poses problems for tissue biopsies. The discrimination of both components could not be performed accurately according to the likelihood of their presence in random biopsies, thus leading to potential underestimations of the frequency of MiNEN diagnosis[15]. Recently, it has been argued that a cutoff value is not mandatory for diagnosing MiNEN because the latest molecular information in the modern classification of these neoplasms has made it possible to demonstrate that both components are clonally related[16].

Another point to be considered is that the determination of NEN on a purely quantitative basis may cause problems in diagnosis, especially with the use of IHC. Indeed, it has been reported that achieving a diagnosis of MiNEN only by quantification with IHC findings has caused inconsistencies and confusion in terminology[17]. Therefore, the WHO has clearly stated that the findings of IHC alone are not sufficient for the diagnosis of MiNEN, and those histopathological findings should be present in each morphological component[9,18].

To date, the definition of MiNEN also excludes nonneuroendocrine neoplasms in which scattered tumor cells express neuroendocrine markers without the presence of neuroendocrine morphology (Table 1). Since neuroendocrine markers can be positive in many nonneuroendocrine tumors, including poorly differentiated adenocarcinomas, performing IHC alone may lead to an overdiagnosis of MiNEN [19-21]. Therefore, it is highly recommended to avoid the application of neuroendocrine markers to tumors that do not have a morphological neuroendocrine component to overcome this difficulty.

Another issue that should be considered is that the diagnosis of MINEN must be given in cases that have not undergone neoadjuvant treatment. Many studies have observed that the number of neuroendocrine cells may increase after treatment (especially chemotherapy) in GIS adenocarcinomas[22-24]. The effective mechanisms of this phenomenon are not fully known and await clarification, and the latest WHO classification does not include these tumors in the MiNEN group.

As pointed out previously, the behavior of MiNEN does not correspond to the average of the two components,such as hybrid neoplasia, but the sum of the two components because each of component can independently progress and metastasize[17,25]. For this reason, it is crucial to detect and evaluate each component separately during pathological examination to determine the treatment of the tumor. Although the nonneuroendocrine component of many MiNENs is more frequently composed of adenocarcinomas, it may differ according to the location within the GIS (see below), which should be

Table 1 Discrimination among mixed neuroendocrine-nonneuroendocrine neoplasms, neuroendocrine tumors, and carcinomas according to pathological parameters

Morphology	Dual differentiation present			Dual differentiation absent		
Immunohistochemisty						
Neuroendocrine markers	(-)	(+)	(+)	(+)	(-)	Few (+) cells[1]
Nonneuroendocrine markers	(+)	Few (+) cells	(+)	(+)	(+)	(+)
Diagnosis	Carcinoma	NEN	MiNEN	Amphicrine tumor	Carcinoma	Carcinoma

[1]These cells frequently express synaptophysin, the expression of other neuroendocrine markers is rare.
NEN: Neuroendocrine neoplasia.

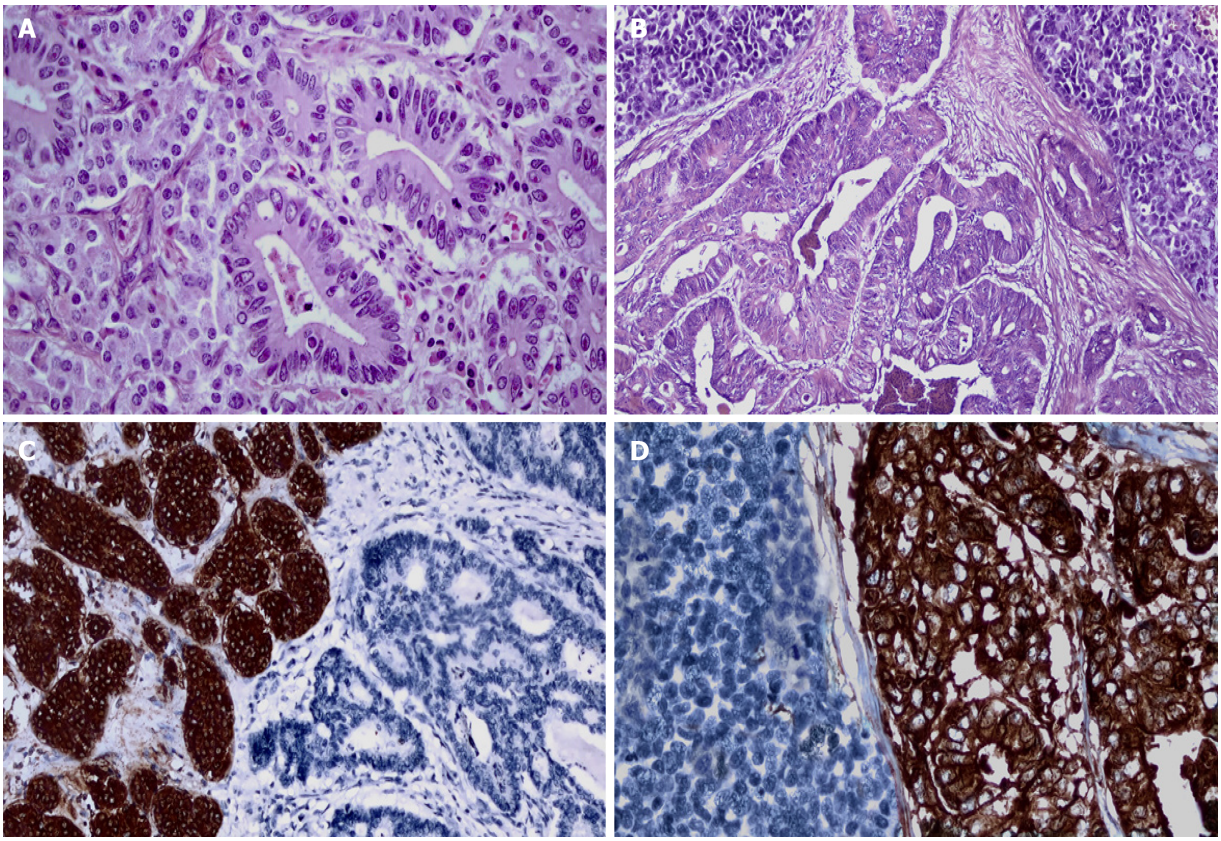


Figure 1 Examples of histopathological and immunohistochemical findings in gastrointestinal mixed neuroendocrine-nonneuroendocrine neoplasms. A: Gastric mixed neuroendocrine-nonneuroendocrine neoplasms (MiNEN) composed of a NET (lower left) intermingled with an adenocarcinoma (x 400); B: Colonic MiNEN constituted from a neuroendocrine carcinoma and an adenocarcinoma (x 200); C. Diffuse immunostaining with synaptophysin in the neuroendocrine component of a colonic MiNEN (x 200); D: The adenocarcinoma component of this MiNEN shows diffuse positivity with CK20 (x 400).

considered during evaluation. This part of the tumor should be graded according to the type of nonneuroendocrine component of MiNEN. Similarly, neuroendocrine components should be evaluated according to the WHO classification and tumors should be graded according to the Ki-67 index percentage and mitotic count.

SUBTYPES OF MiNEN

These tumors are subdivided into three categories. While collision MiNEN is defined as two coexisting cell populations that remain separate without transition, composite MiNEN involves two morphologically distinct components that coexist in an intermingled population[25]. To date, true collision tumors consisting of two independent neoplasms arising in the same organ, even if they abut one another, should not be considered MiNEN unless these components are presumed to be clonally related[18]. The last group includes amphicrine tumors composed of a morphologically one-cell population that displays

the phenotypes of neuroendocrine and adenocarcinoma phenotypes. These cells show coexisting morphological, immunohistochemical, and ultrastructural properties that present both neuroendocrine and exocrine differentiation. Although these tumors have been described for many years in many locations, including the GIS, there is no consensus regarding their relationship with MiNENs. These rare neoplasms have been observed in the stomach, pancreas, appendix, and colon[26-28]. In the appendix, where they are observed relatively more frequently, they are now classified as goblet cell adenocarcinomas[29]. Although this nomenclature does not reflect the amphicrine characteristics of the cells, the term goblet cell carcinoid has been used for many years to avoid misclassification. A recent elegant study by Huang *et al*[30] provided evidence that amphicrine carcinomas arising from the stomach and intestine are distinct tumors with different clinicopathological and pancancer transcriptome features. The latter revealed that although amphicrine neoplasms show similarities to adenocarcinomas, they are not similar to NENs. However, since the nature of NENs was not specified in the study, this finding awaits further investigation. In an elegant study comparing the similarities and differences in genetic alterations between gastric amphicrine carcinomas and MiNENs, Sun *et al*[31] observed that the copy number (CN) characteristics of gastric amphicrine carcinomas were different from those of MiNENs based on a hierarchical clustering analysis, thus supporting that amphicrine carcinoma is a separate entity from MiNENs. In addition, a higher CN level of C5 (complement C5) was observed in amphicrine carcinomas than in MiNENs, suggesting that these tumors might benefit more from C5 inhibitors than MiNENs.

Currently available data also suggest that the inclusion of amphicrine tumors in the definition of MiNEN is not supported based on clinical and pathological features. Moreover, the fact that amphicrine tumors are a subject of debate does not exclude their consideration in the differential diagnosis of MiNEN.

PATHOGENESIS OF MiNEN

Although the carcinogenesis of MiNEN has not been clarified, recent studies suggest that they are derived from a single precursor cell that has the capacity for dual differentiation after the initiation of carcinogenesis. Studies on colorectal MiNEN have demonstrated that both components share common driver genetic aberrations in critical oncogenes and/or their protein products, including tumor protein p53 (TP53), retinoblastoma tumor corepressor 1 (RB1), adenomatous polyposis coli (APC), phosphatase and tensin homolog (PTEN), Kirsten rat sarcoma viral oncogene homolog (KRAS), v-myc avian myelocytomatosis viral oncogene homolog (MYC), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), and v-raf murine sarcoma viral oncogene homolog B (BRAF)[32-36]. Recent data have also suggested that microsatellite instability (MSI) and prostaglandin E2 receptor 4 (PTGER4) activation are also involved during the evolution of MiNEN[35]. Many studies have provided essential data about common driver genetic aberrations[32-34,37,38] . Indeed, both components display loss of heterozygosity (LOH) at multiple loci and mutations in key oncogenes with high allele frequencies compared to exclusive alterations of a single component, thus supporting their common clonal origin hypothesis, at least in the earlier steps of carcinogenesis[32-34,37,38].

In a more recent molecular study in gastric tumors with targeted DNA sequencing, a great majority of mutations were shared by both ADC and NEC components, and among them, TP53 was the most commonly mutated gene (69.2%)[39]. A subset of TP53-wild-type tumors had a microsatellite-unstable phenotype or amplifications in various oncogenes, including ERBB2 and NMYC. While differentially altered genes of ADC components were significantly associated with receptor tyrosine kinase signaling pathways, differentially altered genes of NEC components were significantly associated with the NOTCH signaling pathway, thus providing evidence for a possible clonal origin of ADC and NEC components of MiNENs[39].

Due to additional genetic alterations in poorly differentiated NEC (PDNEC), it is suggested that two different components activate separate genetic pathways at some step of carcinogenesis[32,36,38,40]. Another finding supporting a common carcinogenic pathway is that a proportion of either PDNEC or MiNEN of the stomach and colorectum has increased methylation with a mismatch repair-deficient phenotype[41-44]. Since these tumors had less aggressive behavior, similar to sporadic colon adenocarcinoma in the elderly, it is proposed that mismatch repair deficiency could be a pathway between PDNEC/MiNEN and adenocarcinoma[21,25]. In addition, the neuroendocrine component of PDNEC carries mutations specific to the organ from which it originates and is similar to adenocarcinoma in localization; meanwhile, these alterations are different from a common neuroendocrine alteration. When CDK2A and APC mutations were compared in the PDNEC of the colon and pancreas, it was observed that the CDK2A mutation was higher in pancreatic PDNEC compared with APC mutations, which were higher in the colon; this finding was consistent with adenocarcinomas originating from these organs [45]. The comparison of MiNEN with their pure neuroendocrine counterparts in the colon showed that while the former shares a similar copy number aberration profile with adenocarcinoma, the latter displays different structural aberrations; thus, the developmental pathway suggests that MiNEN is related to the nonneuroendocrine component but not to neuroendocrine carcinomas[34,35]. As noted

above, exclusive alterations in the neuroendocrine component carry a higher number of aberrations and an imbalance of alleles with a more aggressive phenotype, thus leading some authors to suggest that nonneuroendocrine components give rise to the neuroendocrine component through transdifferentiation, where c-myc and SMARC4 are potentially involved[32,46]. A significant increase in the number of neuroendocrine marker-expressing cells following neoadjuvant therapy in PDNEC supports the development of a neuroendocrine component through the adenoma-adenocarcinoma sequence[22,24]. The presence of cases of MiNEN that combine PDNEC and adenoma without adenocarcinoma is also postulated as evidence of a common carcinogenic pathway[47]. Evidence has also been obtained that the two components demonstrate distinct genetic patterns, suggesting that some MiNENs have polyclonal origins[35,37,38]. Interestingly, well-differentiated NET components of MiNEN do not share similar genetic alterations observed in their adenoma/adenocarcinoma counterparts, such as LOH of APC, KRAS, and TP53, although they do display specific alterations that are usually found in NETs (but not PDNECs), such as LOH of VHL, which may also represent true collision MiNEN with an independent carcinogenic pathway[42,47].

MANAGEMENT OF MiNEN

Although there is no complete consensus on the treatment of MiNEN, the presence of tumor metastasis at the time of diagnosis and histopathological MiNEN grading of the tumor play a vital role in the choice of therapeutic options[20]. The classification of MiNEN according to the grade malignancy is presented in Table 2.

Localized MiNEN

In all localized MiNENs, curative-intent surgery is recommended as the first treatment of choice, if available. Even in high-grade MiNENs, because of their less aggressive behavior than pure NECs, tumors with an acinar component of the pancreas belonging to this category have benefited from such treatment[48-50]. In the same group, although combinations of etoposide (VP16) and platinum salt or a combination of 5-fluorouracil (5FU) with irinotecan (IRI)- or oxaliplatin (OX)-based preoperative and postoperative chemotherapy have been used in tumor management similar to PDNEC therapy, the role of adjuvant therapy is still not completely defined[44,51-53]. Moreover, in the intermediate group, recent studies suggest replacing this therapy with a combination of 5FU and IRI and/or OX or gemcitabine (GEM) and/or OX for treatment parallel to the chemotherapy applied to adenocarcinomas, and in some cases, radiotherapy has been used as a treatment option in addition to chemotherapy[3,54-57].

Metastatic MiNEN

Unfortunately, extensive surgery does not seem to be an option in this group because the risk outweighs the benefits, especially in high-grade MiNENs. Therefore, systemic chemotherapy is generally performed according to the type present at metastatic sites. In patients with both components diagnosed either at the metastatic site or the primary tumor, therapy is based on the most aggressive component[3,54-57]. Since some intermediate-grade MiNENs with predominant NET components frequently express type 2 somatostatin receptors, such cases may also benefit from long-acting somatostatin analogs and peptide irradiation nucleotide therapy[58-60]. Although MANET is not categorized as MiNEN, its neuroendocrine component can metastasize. Therefore, pure NET-based chemotherapy and peptide irradiation nucleotide therapy are recommended for their treatment. At present, Akt/mTOR mutations have not been investigated in MiNENs. However, some patients who benefit from everolimus have been described[61].

Despite all these findings, the fact that further studies in large series are needed to define the treatment of patients more precisely with MiNEN should not be overlooked. Future studies aiming to determine the molecular vulnerability of both components in MiNEN cases in the GIS diagnosed based on criteria recommended for the diagnosis of these tumors may allow for the development of targeted therapies against both components and improve their treatment.

The treatment approach in MiNENs in both groups is briefly presented in Tables 3 and 4.

ORGAN-SPECIFIC CLINICOPATHOLOGICAL FINDINGS

Esophagus and gastroesophageal junction

Recently, a comprehensive systematic review and a multicenter study performed by Frizziero *et al*[3,15] showed that esophageal MiNENs accounted for between 5.9% and 15.9% of all GIS MiNENs. These tumors, which account for approximately one-quarter of the NENs observed in this localization, show an apparent male predominance and are observed at advanced ages (6th decade) in the distal third of the esophagus[2,19,62]. Although the tumor consists of NEC and squamous cell carcinoma (SCC) in

Table 2 Classification of mixed neuroendocrine-nonneuroendocrine neoplasms depending on the grade of malignancy[15]

Grade of MiNEN	Nonneuroendocrine component	Endocrine component
High-grade	Adenocarcinoma	PDNEC, NET G3[1]
	Squamous cell carcinoma	
	Cholangiocarcinoma	
	Ductal and acinar cell carcinoma	
	Ductal adenocarcinoma	
High-grade	Acinar cell carcinoma	NET, G1 or G2[1]
	Ductal adenocarcinoma	
	Acinar cell carcinoma	
Intermediate grade	Adenocarcinoma	NET, G1 or G2[1]
Low-grade[2]	Adenoma	NET, G1 or G2

[1]Grading should be performed according to the latest WHO classification.
[2]Those tumors are not included in the mixed neuroendocrine-nonneuroendocrine neoplasms category.
MiNEN: Mixed neuroendocrine-nonneuroendocrine neoplasms; PDNEC: Poorly differentiated neuroendocrine carcinoma; G: Grade; NET: Neuroendocrine tumor.

Table 3 Summary of the therapy protocols applied to localized mixed neuroendocrine-nonneuroendocrine neoplasms

Surgery	Complete resection is possible			Complete resection is not possible		
MiNEN grade	High	Intermediate	Low	High	Intermediate	Low
Chemotherapy	PDNEC-like	ADC-like	NR	PDNEC-like	ADC- like	NET-like

MiNEN: Mixed neuroendocrine-nonneuroendocrine neoplasms; ADC: Adenocarcinoma; PDNEC: Poorly differentiated neuroendocrine carcinoma; NET: Neuroendocrine tumor.

Table 4 Summary of the therapy protocols applied to metastatic mixed neuroendocrine-nonneuroendocrine neoplasms

Metastatic component	Defined		Not defined
	One component	Two components	
Chemotherapy target	The metastatic component	The most aggressive component	The most aggressive component in the primary tumor

most cases, there are rare adenocarcinoma cases, particularly adenocarcinoma, in the background of Barrett's esophagus[40,63,64]. Molecular data indicate their monoclonal origin, as demonstrated by LOH, RB1, TP53, and alterations in TP63, SOX2, DVL3, PTEN, PIK3A, and KRAS[40]. However, the monoclonal origin of esophageal MiNEN with a nonendocrine component composed of adenocarcinoma on the background of Barrett's metaplasia remains to be elucidated, and some authors postulate that this group reflects a true collision tumor rather than MiNEN[64].

In the esophagus, because the discrimination of basaloid SCC and small-cell NEC (SNEC) has paramount importance and may pose a diagnostic pitfall in routine microscopic evaluation, immunohistochemical staining for high molecular weight cytokeratins, p63, and p40, is highly recommended to discriminate basaloid SCC from SCNEC[17].

Unfortunately, their lower metastatic capacity (25% *vs* 54%) and longer survival time (28 *vs* 15 mo) relative to pure PDNEC do not change their poor prognosis[65]. Another important finding is the predictive role of the Ki-67 proliferation index of NEC for prognosis[15,66]. More recently, any statistically significant difference in OS between gastroesophageal GEP MiNEN *vs* colorectal MiNEN was detected[66].

Stomach

These tumors constitute 6%–20% of MiNENs located in the GIS, and 7% of NENs are located in the stomach[14,15,67]. Similar to those in the esophagus, stomach tumors are observed in elderly patients (5^{th}-6^{th} decades) and show a male predominance. Based on the macroscopic appearance of these tumors,

which are observed equally in the corpus and antrum of the stomach, they are not different from adenocarcinomas, and specific diagnostic findings on endoscopy have not been obtained[19]. Most of these aggressive tumors consist of well-differentiated adenocarcinomas, and the PDNEC component is mainly located deeper in the organ[68,69]. Although cases composed of adenocarcinoma and NETs have been recorded, it is suggested that the term MANEC can be retained for MiNEN at this location[19]. MiNENs composed of gastric NEN and adenocarcinoma have been described in the setting of chronic atrophic gastritis, etiological factors have not yet been entirely identified[70]. Recent molecular studies indicated a monoclonal origin[37,44,71]. Ishida *et al*[72] compared the molecular pathology of poorly differentiated NEC and MiNEN of the stomach by whole-exome sequencing. The analysis revealed recurrent mutations in 62% of TP53 cases, and they were more frequent in MiNENs than in NECs. Frameshift mutations of APC were observed in two MiNEN cases. In cases of MiNEN, two histological components shared mutations in TP53, APC, and ZNF521, whereas alterations in CTNNB1, KMT2C, PTEN, and SPEN were observed in neuroendocrine components only. They concluded that TP53 is a single, frequently mutated gene in gastric NEC and MiNEN, and alterations in other genes are less common, thus resembling the mutation profiles of gastric adenocarcinomas. Another interesting previous finding is the presence of ATRX gene mutations (primary partial loss) in 37% of cases involving a substantial proportion of gastric MiNEN[73]. However, these findings should be investigated in further studies. Gastric MiNENs are tumors with a poor prognosis that show lymph node and distant metastases at the time of initial diagnosis, and the prognosis is slightly better than that of pure PDNEC[15,74,75]. Similar to these findings, in a recent study including 401 patients, the 5-year disease-free survival was 51.1%, which was significantly better than that of NEC (47,6%) and worse than that of adenocarcinoma (57,8%). Furthermore, in the same series, advanced stages and lymph node metastasis were independent risk factors related to distant recurrence[76].

Small intestines

MiNENs of the small intestines frequently in the duodenum, where they are mainly located in the ampulla[77-81]. MiNENs of the jejunum and ileum are exceedingly rare, similar to PDNECs encountered in these regions[19]. They are equally observed in both sexes and older patients. While adenocarcinoma constitutes the nonneuroendocrine component of tumors in many cases, rare cases of SCC have also been recorded[77,78]. Since the neuroendocrine component is frequently located in the deeper part of the intestinal wall, they are frequently diagnosed as adenocarcinoma from biopsies[81]. The histological subtype of adenocarcinoma forming these tumors was also found to be associated with tumor behavior. MiNENs with intestinal-type adenocarcinoma have a better prognosis than those with the pancreaticobiliary subtype[20,25]. However, ampullary MiNENs are aggressive tumors with a poor prognosis and generally present at advanced stages[15,17]. This finding contrasts with MiNEN in the other part of the duodenum, which combine intestinal-phenotype adenocarcinoma and a well-differentiated somatostatin-secreting NET[20]. They are mostly superficial and not highly aggressive, and distant metastasis is rare.

Appendix

The last WHO classification of MiNEN indicated that these tumors were composed of two morphologically recognizable components generally represented by adenocarcinoma and NEC, and this classification has provided knowledge about mixed tumors in the appendix[18]. The exclusion of goblet cell carcinoids, which are currently determined to be amphicrine tumors, from the MiNEN group has led to the limited applicability of past clinicopathological data on these tumors located in the appendix, thus necessitating further evaluation of the findings related to this group[29]. A few studies conducted in the recent past indicate that MiNENs constitute 10% of malignancies at this location. On the other hand, a systematic review showed that among the lower gastrointestinal tract organs, these tumors were most frequently (60.3%) localized in the appendix[82]. An interesting finding is that the age-adjusted incidence for MiNENs increased from 0.01/100000 person-years to 0.07/100000 person-years (range 2004-2016), with an annual percentage change (APC) of 13.8%[83]. This finding can be attributed to the increase in clinical recognition and better diagnostic technologies over the years. They are observed in advanced age (between 58-60 years) mostly encountered incidentally and discovered at advanced stages [56,83,84]. Although recent studies indicate that these tumors do not show sex predilection, new findings that APC shows significant differences according to sex (13.81% in females *vs* 12.24% for males) need to be clarified[83].

Their overall survival rate is 6.5 years, which is better than that of signet ring cell carcinomas (2.1 years) and worse than that of goblet cell adenocarcinomas (13.8 years) and pure NETs (39.4 years)[56, 84]. In a more recent study, the prognosis of 315 patients with MiNEN was compared with that of other histological subtypes in the appendix, including NETs, NECs, goblet cell carcinoma, signet ring cell carcinoma, mucinous adenocarcinoma and nonmucinous adenocarcinoma, based on the surveillance, epidemiology and end results program 18 registries. The overall 5-year survival rate was 57.4%, and the level of invasion was the only independent factor influencing tumor behavior. In addition, multivariate analysis demonstrated that the prognosis of MiNENs was worse than that of NETs, NECs, goblet cell carcinoma, and mucinous adenocarcinoma but better than that of nonmucinous adenocarcinoma and signet ring cell carcinoma[83].

Their pathological differential diagnosis should include goblet cell carcinomas, tubular-type carcinoids and pure NETs with glandular configurations.

Colon and rectum

Tumors in this region constitute more than half of all MiNENs of the GIS[67,85]. In particular, evidence has shown that they are observed more frequently than pure NETs in resections performed in this region[85]. Their distribution among NENs is 14-20% and 1%-3% in the colon and rectum, respectively. They are more common in men than women and observed at an advanced age (6th decade)[25,67,85]. Although they do not have specific clinical findings, they have been defined in the background of inflammatory bowel diseases[86-89]. As a result of their more frequent detection in these regions, the number of molecular studies performed in MiNEN exceeds that of other localizations, and they have provided important data regarding their pathogenesis. The same genetic alterations in both components strongly support their monoclonal origin from a common precursor progenitor cell[33,35,37,38,42]. Parallel to these findings, a recent case showed that in addition to microsatellite instability due to MLH1 promoter methylation, the same mutations affecting the *ARID1A*, *ASXL1*, *BLM*, and *RNF43* genes occur in both components, as determined by a multigene next-generation sequencing panel. On the other hand, BRCA2 has been explicitly altered in the neuroendocrine area. Although the latter observation suggested that BRCA2 could be a potential new target for MiNEN, the lack of this alteration in the nonneuroendocrine part of the tumor requires further consideration concerning intratumor heterogeneity[90].

Macroscopically, these tumors form masses (average 5 cm) without distinguishing features from adenocarcinomas. Histologically, most cases are composed of adenocarcinoma and NEC. Rare cases in which the nonneuroendocrine component consisted of squamous cell carcinoma have also been noted [43,91]. If the neuroendocrine component consists of PDNEC, it is consistently observed in metastases. In comparison, adenocarcinomas are observed in one-third of cases[47]. Such tumors show aggressive behavior and have poor prognosis (overall survival: 12.2 mo) according to the Ki67 proliferative index of the NEC component as well as the MSI status and stage[42,43,67]. Recently, a systematic review demonstrated that if the neuroendocrine component consists of NETs, it is unknown which component will be encountered in metastatic sites because of a higher grade, and the predominance of one component does not warrant their presence in metastatic sites[47,92,93]. This finding emphasizes the complexity of MiNENs and the need for an accurate morphological description of all components. A recent systematic review also demonstrated that in MiNENs of the lower gastrointestinal tract, the site of origin in those with metastatic disease at diagnosis appeared to influence prognosis. The median survival time was 12.3 mo for those with primary colonic tumors *vs* 11.7 mo for those with primary anorectal tumors, with hazard ratios of 1.13 *vs* 0.80, respectively[82].

Pancreas

Pancreatic MiNENs are rare tumors in which the nonneuroendocrine component can be formed by ductal or acinar carcinoma[18]. In addition, tumors with a nonneuroendocrine part consisting of ductal and acinar carcinomas are extremely rare and defined as mixed ductal-acinar-neuroendocrine carcinomas[94]. Tumors with mixed ductal–neuroendocrine carcinoma are rare and account for approximately 0.5%–2% of all ductal adenocarcinomas and 5% of all NENs arising from this organ[27]. There is no sex predilection. Although the average age of onset is 68, they are observed in a wide age range, from 21 to 68 years old. They usually consist of NEC accompanying ductal adenocarcinoma[95-97]. They can be located anywhere in the organ and produce clinical symptoms similar to ductal adenocarcinoma without specific clinical findings. Because of the rare nature of these tumors, molecular data are scarce and limited[46,67,98]. Therefore, the diagnosis should be performed using adenocarcinoma and neuroendocrine tumor-specific markers separately and morphologically because they should be clearly distinguished from ductal adenocarcinomas with entrapped islets and NETs with entrapped ductules in the differential diagnosis[17,27]. In the former, the islands present an ovoid shape and have regular contours constituted by endocrine cells without atypia, and express all hormones, which is inconsistent with a predominant cell line of tumor cells; in the latter, however, the absence of atypia of ductal cells , the lack of aberrant P53 staining and the low proliferative index are essential clues in the differential diagnosis (Figure 2). An increase in the number and size of Langerhans islands that accompany chronic obstructive pancreatitis is another diagnostic pitfall in the differential diagnosis. Previous reports indicated that mixed ductal–neuroendocrine carcinomas are aggressive tumors with poor prognoses (5-year survival is 0%)[18,98]. More recently, lymph node metastasis was indicated as an adverse prognostic factor of disease-specific survival in 7 patients with mixed ductal–neuroendocrine carcinomas[99]. Similar findings were also observed by Zhang *et al*[92] in a larger number of patients. Although data for surgically resected cases are very limited in the literature, a cohort study reported that the median survival was 15.3 mo and all cases died due to disease[100]. However, in a recent study evaluating 8 cases with a median follow-up of 21 mo, the overall survival was 88 mo and the 5-year OS was 58%. In addition, the survival of these tumors was better than that of pancreatic ductal adenocarcinomas; thus, further investigation is warranted[101].

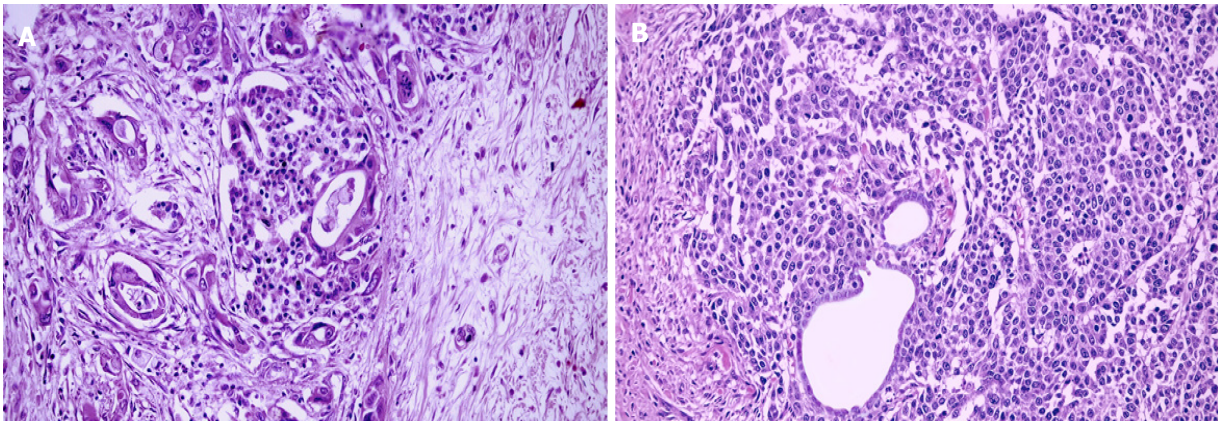


Figure 2 Histopathological pitfalls in the diagnosis of mixed neuroendocrine-nonneuroendocrine neoplasms of the pancreas. A: A ductal adenocarcinoma of the pancreas surrounding and invading an islet in the background of chronic pancreatitis (x 200). The islet has regular contours despite an invasion; B: A neuroendocrine tumor of the pancreas with entrapped two ductulus without atypia. Such areas should be evaluated carefully to avoid a misdiagnosis of mixed neuroendocrine-nonneuroendocrine neoplasms (x 200).

Cases in which the neuroendocrine component consists of NETs have been reported, although their morphology resembles true collision tumors and their monoclonal origin remains to be proven[95,96].

Mixed acinar–neuroendocrine carcinomas are rare and account for nearly one-fifth of all pancreatic ACCs[49,94]. The features of these tumors do not differ from the macroscopic features of ACC, and the tumors are quite large. In the differential diagnosis, morphological and IHC findings should be evaluated together, such as in ductal carcinoma. It is worth noting that 20%-30% of ACCs show a small neuroendocrine cell population, and this morphologically undetected component should not lead to the diagnosis of MiNEN[49]. Immunohistochemical staining with trypsin and bcl-10 [monoclonal antibody directed against the C-terminal portion of bcl-10 (clone 331.3)] is very useful for identifying the acinar component[102] (Figure 3). However, it should be kept in mind that a significant portion of the ACC is stained with synaptophysin.

Moreover, since pure NETs of the pancreas have a better prognosis than these tumors, advanced immunohistochemical evaluations with bcl-10 and trypsin are recommended for all tumors with neuroendocrine-appearing pancreatic neoplasms that show a high mitotic index, abundant necrosis, and evident nucleoli[17]. Although these tumors seem to share the genetic changes observed in pure ACCs, they do not show characteristic mutations that can be found in pancreatic NETs[103,104].

A recent study suggested that c-MYC alterations are involved in mechanisms leading to the neuroendocrine differentiation of ACCs[46]. Surgical resection and tumor stage are the most important prognostic factors, and the reported 5-year survival rate is 30%–50% for patients who undergo surgery [49,105].

Liver

The nonneuroendocrine component of MiNENs is mostly hepatocellular carcinoma (HCC) and less frequently cholangiocellular carcinoma, and they are rare liver tumors encountered at advanced ages (43-84 years) predominantly in men[2,106,107]. The neuroendocrine components of these tumors are predominantly NECs, and they have a dismal prognosis, with many cases presenting distant metastasis at the time of diagnosis[2,108,109]. More recently, the 1-year cumulative survival rate of patients was reported to be 53%[107].Although the pathogenesis has not been fully elucidated; neuroendocrine differentiation from existing HCC has been suggested[108,109].

Gallbladder and biliary tract

MiNENs of the gallbladder and biliary tract account for 10% of all biliary carcinomas and 2% of all hepatobiliary carcinomas[110,111]. Although considered rare, adenocarcinoma is detected in approximately 30% of NENs, particularly in the gallbladder, and these tumors constitute 35% of NENs in this region, thus indicating that they are more frequent than previously described[79,110]. While the age range is relatively wider than that of many MiNENs, these tumors are observed at an advanced age (mean: 65 years), which is similar to those in other regions of the GIS. However, compared with the male dominance observed in other MiNENs, these tumors are more common in females[18]. The close relationships between MiNENs and inflammatory diseases in these locations suggest that inflammation plays a role in pathogenesis. Recent findings indicate that the NEC component of the tumor is composed of large cell NECs in a great majority of cases (59%), and NECs are incidentally discovered during imaging studies without any specific clinical findings[112]. In parallel, specific findings that differ from the findings for adenocarcinomas on macroscopic examination have not been observed. Although a considerable portion of the tumors are confined to the gallbladder wall at the time of

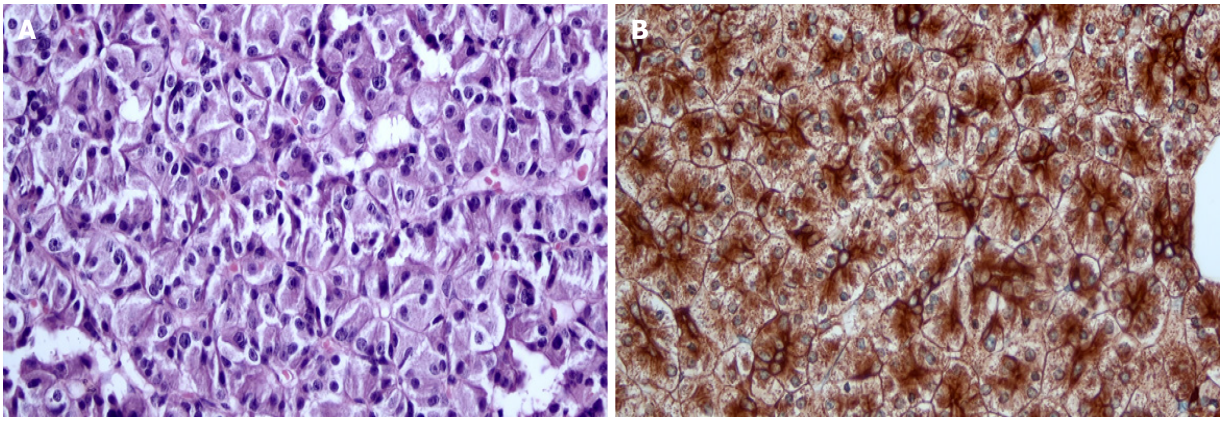


Figure 3 Acinar carcinoma of the pancreas. A: The tumor is composed of cells that demonstrate the presence of monomorphic nuclei, sometimes forming minute lumens. Tumor cells are in a monolayer with basally located nuclei and have a granular eosinophilic cytoplasm (x 400); B: Bcl-10 expression with higher staining in the apical portion of tumor cells (x 400).

diagnosis, one-fifth of the cases have serosa and one-third have adjacent organ invasion[16]. Therefore, the rarity of distant metastases in these tumors does not exclude the possibility that half of them metastasize to neighboring organs (liver, peritoneum, lymph nodes) at the time of diagnosis. On histopathologic evaluation, the nonneuroendocrine component often consists of adenocarcinoma and rarely consists of squamous cell carcinoma or carcinoma with sarcomatous or osteosarcomatous differentiation[112,113]. Few cases with intracystic papillary neoplasms (IPNs) have also been reported[16]. The NEN component, which is usually more deeply located, often consists of NEC. Molecular studies support that MiNENs in this region also consist of a common precursor[16,114]. Although studies on IPN have indicated that the endocrine component originates from these areas, further studies are needed to support this finding. The one-year survival was four times higher in patients with organ-confined tumors than in those with distant metastases, revealing that distant metastasis is the most effective predictive parameter for the course of the disease, thus emphasizing the importance of staging [79,110]. A recent systemic review based on 53 studies to predict the clinicopathological features and prognosis of biliary MiNENs, including gallbladder MiNENs, showed a median overall survival time of 21 mo. In addition, radical resection and small morphological subtype were independent prognostic factors associated with higher overall survival, and radical resection (R0) and younger age (< 65 years) were associated with higher recurrence-free survival time[115].

CONCLUSION

In conclusion, MiNENs of the GIS are a rare group of heterogeneous and aggressive tumors that should be diagnosed in patients without neoadjuvant therapy. Morphological findings are indispensable for their histopathological diagnosis. Evaluations based solely on the percentage of cells stained with IHC may lead to overdiagnosis. Since the current therapeutic approach depends on the grade of MiNEN, each component should be evaluated and graded separately. Although many studies support that these tumors are monoclonal, at least in the early stages of carcinogenesis, these data require additional research support.

Similarly, the 30% cutoff value should be reaffirmed by systematic studies because the possibility of the negative influence of a small component of high-grade NEN on tumor behavior should not be ignored. The deep localization of the NEN component in many organs is another potential limitation leading to their underestimation in biopsies. As recent findings suggest that amphicrine tumors may belong to a different tumor category, more studies are needed to reach a complete conclusion regarding these tumors.

In summary, new cases diagnosed as MiNENs in the GIS according to the currently proposed categories will increase awareness of these tumors, provide new data and eliminate diagnostic controversies of the past.

FOOTNOTES

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ORCID number: Gulsum Ozlem Elpek 0000-0002-1237-5454.

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ORIGINAL ARTICLE

Basic Study

Mucosal bacterial dysbiosis in patients with nodular lymphoid hyperplasia in the terminal ileum

Qiao-Li Jiang, You Lu, Meng-Jie Zhang, Zhen-Yu Cui, Zhong-Mei Pei, Wen-Hua Li, Lun-Gen Lu, Jing-Jing Wang, Ying-Ying Lu

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Qiao-Li Jiang, You Lu, Meng-Jie Zhang, Zhen-Yu Cui, Zhong-Mei Pei, Wen-Hua Li, Ying-Ying Lu, Department of Gastroenterology, Jiading Branch of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201803, China

Lun-Gen Lu, Ying-Ying Lu, Department of Gastroenterology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China

Jing-Jing Wang, Shanghai Key Laboratory of Pancreatic Diseases, Institute of Translational Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China

Corresponding author: Ying-Ying Lu, PhD, Associate Chief Physician, Department of Gastroenterology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 650 Songjiang Road, Shanghai 201620, China. le_voyageur@qq.com

Abstract

BACKGROUND

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the intestinal surface. Patients with terminal ileal NLH may experience long-term abdominal pain, diarrhea, and abdominal distension, among other symptoms. Supplementation with probiotics could mitigate these symptoms. NLH is linked to the immune system, and it may result from accumulation of plasma-cell precursors due to a maturational defect during the development of B lymphocytes. The intestinal microbiome plays an essential role in the immune system. Thus, we speculate that the gut flora plays a key role in terminal ileal NLH.

AIM

To explore the correlation between intestinal flora and terminal ileal NLH.

METHODS

We collected mucosal biopsy samples that were obtained *via* colonoscopy from 15 patients with terminal ileal NLH (the test group) and 15 normal subjects (the control group). We subsequently performed 16S-rRNA gene amplicon sequencing of these samples, and the results were evaluated using alpha diversity, beta diversity and microbial composition analyses. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used to predict the

metabolic pathways and orthologous groups according to the Kyoto Encyclopedia of Genes and Genomes database.

RESULTS

Compared with the control group, the terminal ileal NLH group showed an increased alpha diversity ($P < 0.05$). The overall intestinal microbiota in the NLH group was significantly different from that of the control group ($P < 0.05$), implying that there was the dysbiosis in the terminal ileal NLH patients. The relative abundance of phylum Bacteroidetes was significantly lower in the NLH group, while that of Patescibacteria and Campilobacterota was significantly higher. The genus *Bacteroides* was the dominant gut microbiota in both groups, but its abundance was significantly lower in the test group than it was in the control group. Conversely, the relative abundances of *Haemophilus, Streptococcus, Pseudomonas, Actinomyces, TM7X, Fusobacterium nucleatum, Parvimonas, Granulicatella, Helicobacter*, and the [*Eubacterium*] *nodatum group* were significantly higher in the test group than they were in the control group. In addition, several altered metabolic pathways, orthologous groups, and modules were found. For example, the Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis were both increased in the test group.

CONCLUSION

Maintaining the microbial balance and supplementing targeted protective bacteria could improve symptoms and potentially reduce the risk of lymphoma transformation in patients with terminal ileal NLH.

Key Words: Hyperplasia; Bacteroides; Small intestine; Microbiome; Helicobacter pylori; Colonoscopy

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Core Tip: Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the surface of the intestine. To explore the correlation between the intestinal flora and terminal ileal NLH, we performed bacterial 16S rRNA gene sequencing of mucosal samples from patients with terminal ileal NLH. Our results reveal that specific microflora may act on the mucosa of the small intestine and cause terminal ileal NLH. Therefore, maintaining the balance of intestinal flora and supplementing targeted protective bacteria may improve terminal ileal NLH symptoms and potentially reduce the risk of lymphoma transformation.

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INTRODUCTION

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion, and its incidence has not yet been determined. It is characterized by multiple small nodules on the surface of the intestine, which are found to be present in the lamina propria and superficial submucosa of the intestine[1]. The diagnosis of NLH is mainly based on endoscopic and histological examinations, markedly including the presence of hyperplastic lymphoid follicles and mitotically active germinal centers with well-defined lymphocytic mantles[2]. Terminal ileal NLH is a type of NLH, and patients with terminal ileal NLH demonstrate multiple symptoms that seriously reduce the individual's quality of life, such as chronic diarrhea, abdominal pain, hematochezia, anemia, hypoproteinemia, among other symptoms[3]. Patients with irritable bowel syndrome are more likely to accompany with NLH[4]. During the clinical diagnosis and treatment of patients with terminal ileal NLH, we found that probiotics could improve gastrointestinal symptoms.

NLH may be associated with a risk factor for intestinal lymphoma, as the resolution of gastrointestinal tract nodular lymphoid hyperplasia has been shown following chemotherapy for extraintestinal lymphoma[5]. However, the pathogenesis of NLH has not been fully elucidated yet. A frequently proposed hypothesis implicates an intestinal antigenic trigger, possibly infectious, that leads to the repetitive stimulation and eventual hyperplasia of the lymphoid follicles, which may originate

from proliferative plasma cell precursors related to a maturational defect during the development of B lymphocytes[6]. NLH has been reported in patients with human immunodeficiency virus, common variable immunodeficiency, Giardia lamblia infection, helicobacter pylori (*H.pylori*) infection, familial adenomatous polyposis, and Gardner's syndrome[7]. The intestinal microbiome plays an essential role in the immune system; gut microbiota that colonize the human intestinal tract form a mutual symbiotic relationship with the host and play a key role in regulating the host immune system and metabolism[8, 9]. Conventionally, alterations in the gut microbiota are closely related to inflammatory bowel disease, obesity, colonic adenoma, and colorecatal cancer[10-13], but whether the gut flora plays a role in NLH is unclear.

In this study, we performed bacterial 16S rRNA gene amplicon sequencing of mucosal tissue samples to study the gut microflora dysbiosis that is associated with terminal ileal NLH to determine what alterations occur in microflora and explore the correlation between the intestinal microflora and terminal ileal NLH. Moreover, we used molecular bioinformatic technology to predict the metabolic pathways that are involved in terminal ileal NLH, which will provide the possibility for further targeted intervention therapy.

MATERIALS AND METHODS

Clinical trial design and sampling

A total of 30 patients who underwent a colonoscopy in the Digestive Endoscopy Center at Jiading Branch of Shanghai General Hospital (Shanghai, China) from January 2021 to April 2021 were recruited for this study. A total of 15 Patients with terminal ileal NLH (11 males and 4 females aged 24-44 years)were assigned to the test group, while 15 healthy volunteers (7 males and 8 females aged 30-44 years)were assigned to the control group after undergoing a routine physical examination. There were no statistically significant differences in the general data between the groups ($P > 0.05$). Among the 15 patients with terminal ileal NLH, the most common symptom was diarrhea, followed by abdominal pain and abdominal distension.

Endoscopic images were reviewed and confirmed by the endoscopic team. Terminal ileal NLH was diagnosed using endoscopy and histopathology (Figure 1). We confirmed that there was no history of antibiotic or probiotic administration within the previous two months for all the subjects. Patients with diabetes mellitus, inflammatory bowel disease, previous colon resection, colorectal cancer, or a body mass index ≥30 kg/m^{2}were excluded. This research was approved by the research ethics boards of Shanghai General Hospital (2021KY085), and written informed consent was obtained from all the patients before sample collection. We collected mucosal biopsy samples that were obtained *via* colonoscopy from both groups. All samples were frozen immediately after sampling and stored at -80 °C.

DNA extraction/isolation

Microbial genomic DNA was extracted from each sample using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc., GA) according to the manufacturer's instructions. The samples were suspended in 790 μl of sterile lysis buffer (4M guanidine thiocyanate; 10% N-lauroyl sarcosine; and 5% N-lauroyl sarcosine-0.1 M phosphate buffer [pH, 8.0]) in a 2-ml screw-cap tube containing 1 gof glass beads (0.1mm BioSpec Products, Inc., United States). This mixture was vortexed vigorously and subsequently incubated at 70°C for 1 h. After incubation by bead beating for 10 min at maximum speed, the extracted DNA was stored at -20 °C for further analysis.

PCR amplification

The V3-V4 region of the bacterial 16S ribosomal RNA gene from each sample was amplified using the universal bacterial primers F1 and R2 (5′-CCTACGGGNGGCWGCAG-3′and 5′-GACTACHVGGGTATCTAATCC-3′); these primers correspond to positions 341 to 805 in the Escherichia coli 16S rRNA gene. The PCR reactions were run in a T100™ Thermal Cycler PCR system (Bio-Rad Laboratories, Inc., United States) using the following protocol: 3 min of denaturation at 95 °C, followed by 21 0.5-min denaturation cycles at 94 °C, 0.5 min of annealing at 54 °C, and 0.5 min of elongation at 72 °C, with a final 5 min extension at 72 °C.

Sequencing

The amplicons from different samples were purified using Hieff NGS® DNA Selection Beads (Yeasen-Biotech Co., Ltd., Shanghai, China). The products were indexed and mixed at equal ratios for sequencing by Shanghai Mobio Biomedical Technology Co., Ltd. using the Miseq platform (Illumina Inc., United States) according to the manufacturer's instructions.

Data availability

The raw sequencing data from the 16S rRNA gene V3-V4 regions and the accompanying information

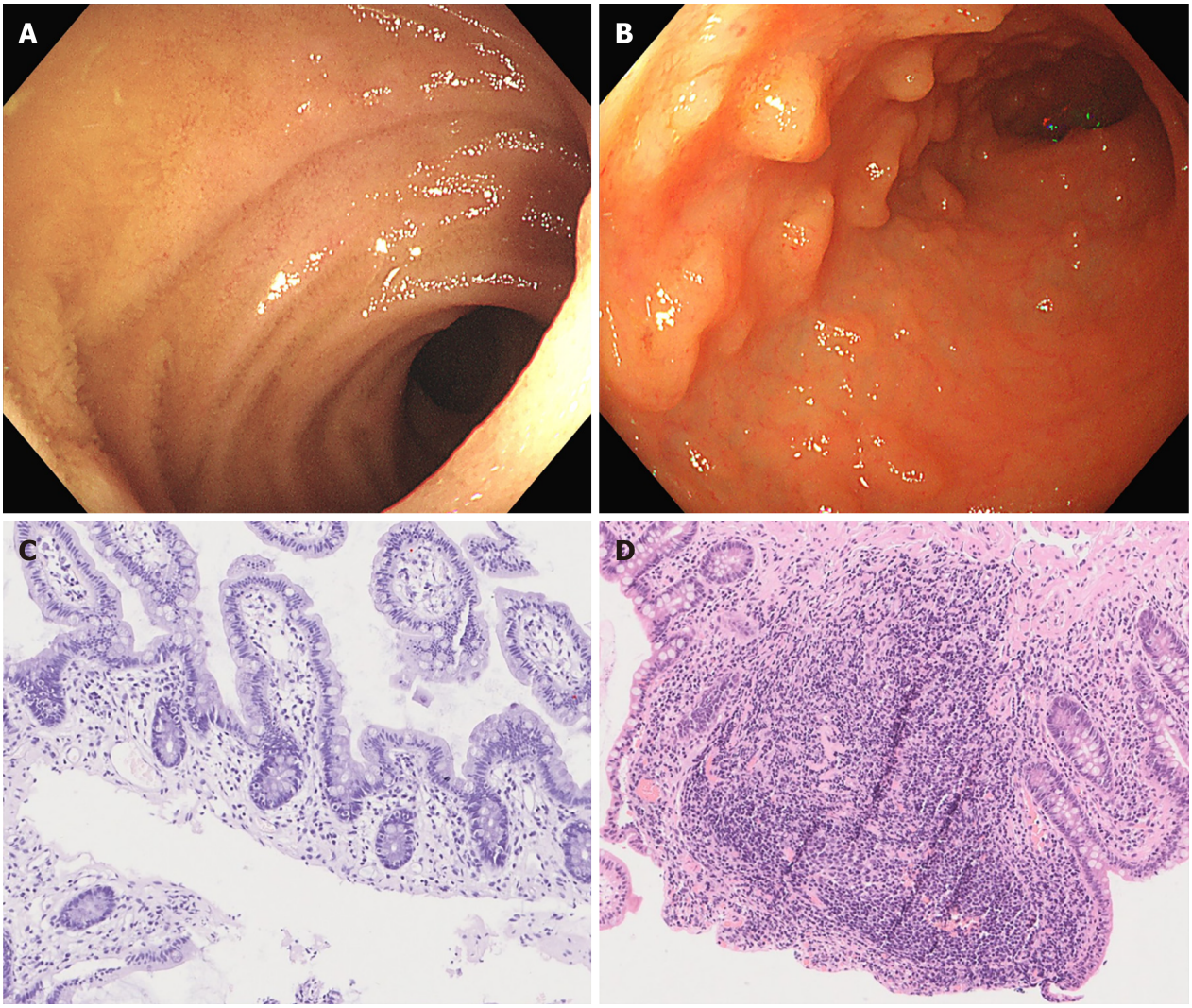


Figure 1 Endoscopic images and pathological characteristics of nodular lymphoid hyperplasia and normal mucosa in the terminal ileum.
A: Endoscopic image of a normal terminal ileum from a healthy volunteer; B: Endoscopic image of nodular lymphoid hyperplasia (NLH) in terminal ileum of a patients; C: Pathological characteristics of normal terminal ileal mucosa (×100; scale bar, 200 μm); D: Pathological characteristics of terminal ileal NLH: hyperplasic lymphoid follicles germinal centers (×100; scale bar, 200 μm).

are available in the Sequence Read Archive database under accession number PRJNA759383.

Bioinformatics analysis

Clean data was extracted from the raw data using USEARCH version 11.0.667 (http://www.drive5.com/usearch/). Quality-filtered sequences were clustered into unique sequences and sorted in order of decreasing abundance to identify representative sequences using UPARSE according to the UPARSE Operational Taxonomic Units (OTUs) analysis pipeline, with singletons being omitted. OTUs were classified based on a 97% similarity after the chimeric sequences were removed using UPARSE version 7.1 (http://drive5.com/uparse/), after which they were annotated using the SILVA reference database (SSU138). The number of common OTUs in both groups was calculated and the results were shown using a Venn diagram.

Alpha diversity, which reflects the diversity of microbiome community, was obtained by analyzing the ACE estimator, Chao 1 estimator, Shannon-Wiener diversity index, and Simpson diversity index using Mothur version 1.42.1. The larger the Chao 1 or ACE index, the higher the gut flora abundance, whereas the higher the Shannon or Simpson index, the higher the community diversity.

To visualize the structural diversity of the gut microbiome in the discovery group, we used a principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) plots based on the Bray-Curtis distances. The corresponding statistical significance of the beta diversity was measured separately using an Adonis analysis.

To compare the microbial communities at each taxonomic level between the groups, significant between-group differences in the microbial composition were analyzed using a Wilcoxon rank-sum test. A linear discriminant analysis effect size (LEfSe) was used to show the maximum difference in the microbial structures between the groups (LEfSe version 1.1, https://github.com/SegataLab/Lefse) to

determine the specific bacterial taxa and predominant bacteria that are related to terminal ileal NLH. The results of the microbiome heatmap analysis, as provided by a random forests model, revealed a discriminatory intestinal microbiome between the two groups.

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2 version 2.4.1 (https://github.com/picrust/picrust2/wiki) was used to predict different metabolic pathways and orthologous groups between the groups according to the yoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

Non-parametric Mann-Whitney U tests were used to determine if there were significant differences between the groups. A Student's t-test was performed using SPSS for Windows, version 20.

RESULTS

OTU clustering and alpha diversity analysis of the mucosal intestinal microflora

In total, 1530914 usable sequences were obtained from 30 samples using Illumina high-throughput sequencing technology. From these, 1263179 high-quality sequences were selected, with an average of 42106 sequences per sample. Using 97% as the similarity cutoff, we generated 554 OTUs. The Venn diagram showed that 500 of the 554 OTUs were shared by both groups, whereas 33 OTUs were unique to the test group, and 21 were specific to the control group (Figure 2A).

The alpha diversity of the intestinal mucosal microflora was higher for the test group than it was for the control group. As estimated by the observed OTUs in each sample and the ACE and Chao indexes, which reflect the richness of the species diversity, the microbial diversity was significantly higher in the test group than it was in the control group ($P < 0.05$).The index values for the alpha diversity analysis are shown in Figures 2B-F.

The resulting rarefaction curves indicate that the microbial richness of the sampled guts was near saturation at the applied sequencing depth, which was sufficient to identify most of the bacterial community members in each individual. The Shannon-Wiener curve based on the OTUs was already flat, indicating that our sequencing depth was already adequate. The specaccum species accumulation curves revealed that the OTU richness approached saturation in all the samples (Supplementary Figure 1).

Analysis of the beta diversity based on OTU levels

To evaluate the similarities between all samples, the ecologic distances, which were calculated based on the Bray-Curtis distances, were visualized using a PCoA plot. A certain tendency of separation was found between both groups, indicating that the bacterial flora differences in the overall structure of gut microbiota existed between the groups (Figure 3A). Moreover, a non-metric multidimensional scaling analysis based on the Bray-Curtis distances showed a significant difference in gut microbiomes between both groups (Figure 3B).

Additionally, an Adonis analysis showed that there was a significant difference between the two groups ($P < 0.05$). Based on the unweighted and weighted UniFrac distances, a PCoA also showed that the microbial composition of the test group deviated from the control group (Adonis: $P = 0.0142$ and $P = 0.0467$, respectively).

Flora composition in the two sample groups

A total of 19 phyla were detected by classifying the species of all OTUs in the terminal ileal mucosa. At the phylum level, the gut microbiota of both groups was dominated by Bacteroidetes and Firmicutes, followed by (on average) Proteobacteria. The proportions of dominant flora in the test group were 35.19%, 39.29%, and 14.31% respectively, while those of the control group were 46.11%, 36.68%, and 8.34%, respectively. The average relative abundance of the microbiome at the phylum level is shown in Figure 4A.

At the genus level, the gut microbiota was dominated by *Bacteroides* in the control group, followed by *Faecalibacterium*, *Fusobacterium*, *Escherichia-Shigella*, and *Prevotella* with proportions of 36.85%, 8.47%, 6.93%, 4.96%, and 4.33%, respectively. Correspondingly, *Bacteroides* was the most dominant bacteria in the test group, followed by *Prevotella*,*Faecalibacterium*, *Fusobacterium*, and Escherichia−Shigella, with proportions of 19.63%, 10.31%, 8.39%, 8.33%, and 7.43%, respectively. The average relative abundance of the microbiome at the genus level is shown in Figure 4B.

Differences in microbiome compositions between the groups

There were significant differences in the microbial composition between the groups, as analyzed by the Wilcoxon rank-sum test. At the phylum level, Bacteroidetes were significantly lower in the test group than in the control group. In contrast, Patescibacteria and Campilobacterota were significantly higher in the test group than in the control group. At the genus level, the number of *Bacteroides* was significantly

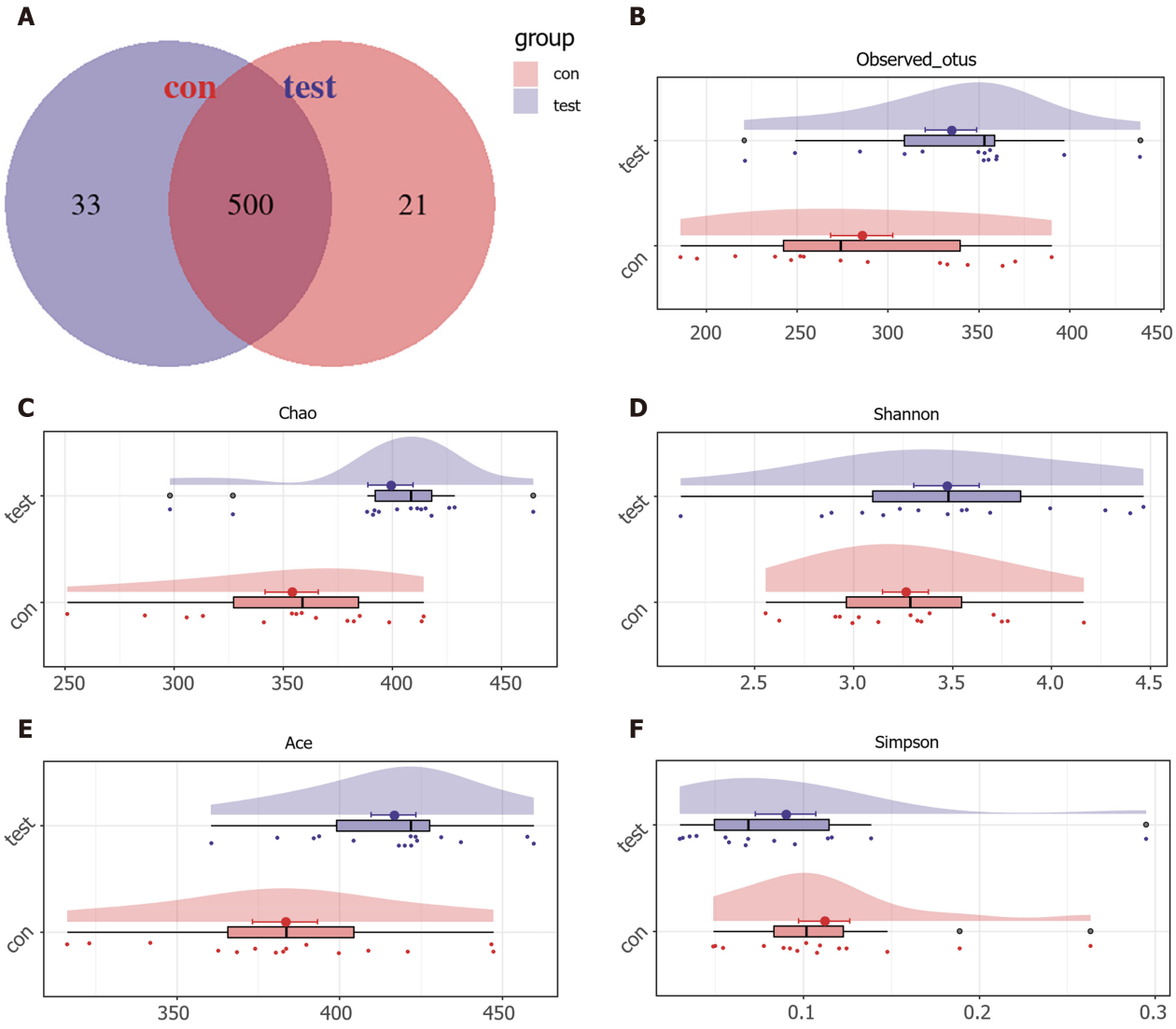


Figure 2 Operational Taxonomic Units clustering and alpha diversity analysis of mucosal intestinal microflora. A: Venn diagram demonstrates the shared and unique Operational Taxonomic Units (OTUs) in both groups; B: The OTUs in the single sample from each group; C: Chao index; D: Shannon-Wiener diversity index; E: ACE estimator; F: Simpson diversity index.

lower in the test group than in the control group. Conversely, the abundances of *Haemophilus*, *Streptococcus*, *Pseudomonas*, *Actinomyces*, *TM7X*, *Parvimonas*, *Granulicatella*, *Helicobacter* and the *[Eubacterium] nodatum group*, among others, were significantly higher in the test group than in the control group (Figures 4C and D).

A LEfSe was used to show the maximum difference in microbial structures between the groups to determine the specific bacterial taxa and predominant bacteria in the patients with terminal ileal NLH. This analysis showed that the abundances of various genera, including *Haemophilus*, *Streptococcus*, *Phocea*, *Candidatus_saccharimonas*, *Pseudomonas*, *Vagococcus*, *Cutibacterium*, *Actinomyces*, the *Eubacterium nodatum group*, *TM7X*, *Delftia*, *Chryseobacterium*, *Peptostreptococcus*, *Helicobacter*, *Parvimonas*, *Solobacterium*, and *Peptococcus*, among others, were significantly higher in the test group than in the control group. Conversely, the abundances of *Bacteroides*, *Bilophila*, and the *Eubacterium hallii group* were significantly higher in the control group than in the test group (Figure 4E).

The results of the heatmap analysis of the microbiomes using a random forest model revealed a discriminatory intestinal microbiome between both groups. A total of 28 OTUs were found to be different between the sample groups. Among these OTUs, 24 were more abundant in the test group than in the control group; these OTUs belonged to the genera of *Rothia*, *Roseburia*, *Cutibacterium*, *Peptococcus*, *Parabacteroides*, *Lachnoanaerobaculum*, *Actinomyces*, *Streptococcus* and *SaccharimonAdales*, *Solobacterium*, *Peptostreptococcus*, *Granulicatella*, *Parvimonas*, *Lachnoclostridium*, *Pseudomonas*, *Fusobacterium* , *Haemophilus*, *Prevotella*, and *Clostridia UCG-014*. *Bacteroides*, *Bilophila*, and the [*Eubacterium*] *Siraeum group* were more abundant in the control group than in the test group (Figure 4F).

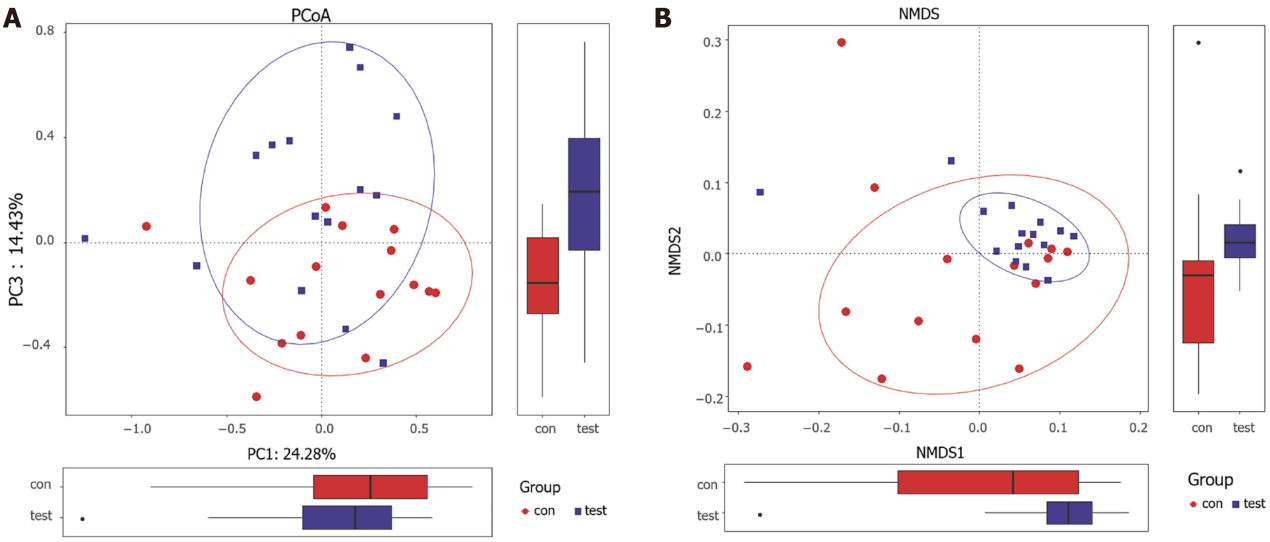


Figure 3 Analysis of beta diversity based on Operational Taxonomic Units levels. A: Principal coordinates analysis plots based on the Bray-Curtis distances shows the differences in the bacterial flora between the terminal ileal nodular lymphoid hyperplasia and control groups; B: Nonmetric multidimensional scaling analysis based on the Bray-Curtis distances shows the differences between both groups. Each symbol represents one sample.

Functional alterations of gut microbiomes in both groups

PICRUSt2 version 2.4.1 was used to predict metabolic pathways and orthologous groups according to the KEGG database, and a LEfSe was subsequently used to sort the different metabolic pathways and orthologous groups between the two groups. The results demonstrated that Photosynthesis, D Alanine metabolism, C5 Branched dibasic acid metabolism, Peptidoglycan biosynthesis, Aminoacyl tRNA biosynthesis, Bacterial chemotaxis, ABC transporters, D glutamine and D glutamate metabolism, synthesis and degradation of ketone bodies, ulfur relay system, ribosome, mismatch repair, homologous recombination, Glycerophospholipid metabolism, base excision repair, DNA replication, butanoate metabolism, bacterial secretion system, terpenoid backbone biosynthesis, and styrene degradation were significantly higher in test group compared to the control group. However, glycosaminoglycan degradation, secondary bile acid biosynthesis, sphingolipid metabolism, biotin metabolism, pentose and glucuronate interconversions, galactose metabolism, streptomycin biosynthesis, cyanoamino acid metabolism, and alanine aspartate and glutamate metabolism pathways were all significantly higher in the control group than in the test group. Altered metabolic pathways, orthologous groups, and modules in both groups are presented in Figure 5.

DISCUSSION

Terminal ileal NLH is commonly detected through colonoscopy. The pathogenesis of terminal ileal NLH remains unclear, it is generally regarded that infection-induced immune responses play a key role. Alterations of the gut microbiota are closely related to immune-related diseases. However, whether the gut flora plays a role in terminal ileal NLH is unclear. Currently, there are no studies that have reported on the relationship between the intestinal flora and terminal ileal NLH. In this study, alpha diversity was higher in the test group than in the control group. *Helicobacter, Fusobacterium nucleatum, Actinomyces, TM7X*, and *Peptostreptococcus* were significantly more abundant in the test group than in the control group. Additionally, the Peptidoglycan and Aminoacyl tRNA biosynthesis pathways were significantly more abundant in the test group than in the control group.

In this study, we found that the bacterial diversity was significantly higher in the terminal ileal NLH group than in the control group, suggesting the presence of a small intestinal bacterial overgrowth (SIBO). SIBO is defined as bacterial overgrowth in the small intestine caused by an abnormally high number of bacteria and/or changes in the kinds of bacteria; it is accompanied by an overgrowth of bacteria in the small bowel in excess of 10^5 colony forming units per milliliter in upper gut aspirate culture[14]. This is due to the bacteria migrating into the small intestine from distal intestinal tract, resulting in intestinal mucosal inflammation and permeability and villi damage, which mainly manifests as nutrient malabsorption, abdominal pain and distension, diarrhea, and intestinal motility abnormity[15]. SIBO is closely associated with many diseases, including colorectal cancer, irritable bowel syndrome, inflammatory bowel disease, and non-alcoholic fatty liver disease[16-19]. In this study, we found an increased alpha diversity of intestinal flora in the test group than in the control group. This observation may be related to the local inflammatory response caused by the overgrowth of intestinal

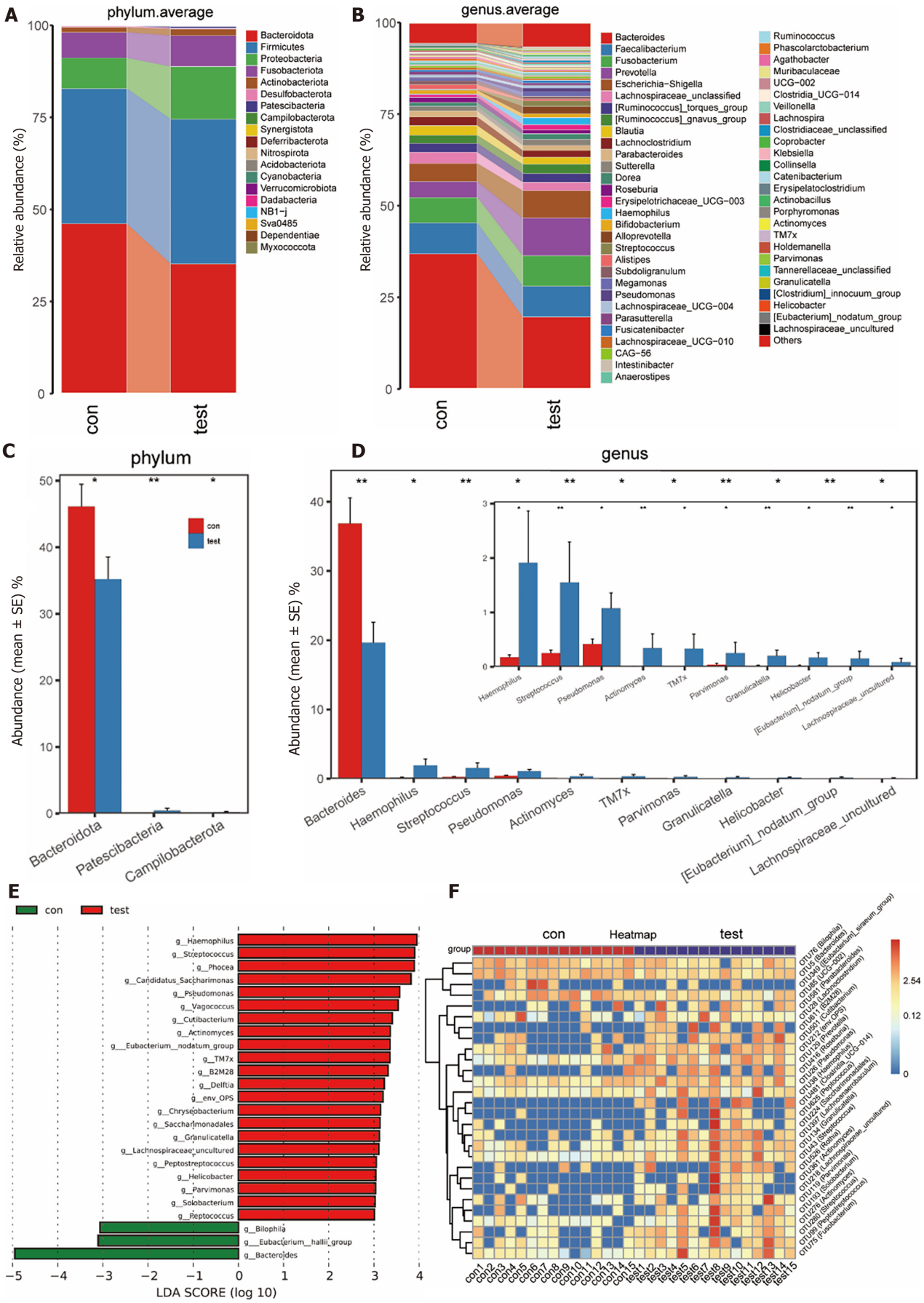


Figure 4 Flora composition and comparison of microbiome composition in both groups. A: Flora composition at the phylum level; B: Flora composition at the genus level; C: The abundance of Bacteroidetes was significantly lower at the phylum level in the test group, as assessed by a Wilcoxon rank-sum test; D: Differences in the flora at the genus level between the groups, as assessed by a Wilcoxon rank-sum test; E: Flora differences at the genus level, as assessed

by Linear discriminant analysis effect size; F: A heatmap analysis of the microbiomes using a random forest model.

bacteria, which results in NLH.

Bacteroidetes and Firmicutes make up most of the human intestinal flora, with a higher abundance of Bacteroidetes. In this study, Bacteroidetes and Firmicutes were the dominant bacteria in both groups, followed by Proteobacteria. Similarly, Bacteroides was the most dominant bacteria in both groups at the genus level. However, Bacteroidetes and Bacteroides were significantly less abundant in the test group than in the control groups, suggesting that Bacteroides may play a protective role in the development of terminal ileal inflammation and NLH.

In our research, *H.pylori* was higher in abundance in the test group. *H.pylori*, which is a proteobacteria, is considered to be a carcinogenic factor of gastric cancer. Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is related to *H.pylori*[20,21], as most patients with MALT can achieve long-term clinical remission after *H.pylori* eradication[22,23]. *H.pylori* is thought to be an antigenic stimulant that can activate the NF-κB pathway[24] and induce pro-inflammatory cytokines expression [25,26]. Persistent inflammation promotes the formation of mucosal lymphoid follicles, typically consisting of B lymphocytes, which might contribute to the genesis of gastric MALT lymphoma once the inflammatory courses are uncontrolled[27]. Khuroo *et al* studied a large cohort of patients (n = 40) with NLH that was etiologically related to *H.pylori* infection. Compared with patients with consistent *H.pylori* infection, patients with eradicated *H.pylori* showed a significant clinical response and lesion regression/resolution [28]. However, the location was limited to the postbulbar duodenum (second and third parts) and duodenojejunal junction in these cases. In our study, the *H.pylori* abundance increased in test group. Moreover, it has been reported that NLH may be associated with an increased risk of parenteral lymphoma. However, currently, there are no relevant reports on the correlation and causal relationship between terminal ileal NLH and *H.pylori*, which is worthy of studying. When treating patients with *H.pylori*, we suggest that endoscopists should routinely observe the terminal ileum.

In this study, the abundance of Actinomycetes and TM7x increased in the test group. Actinomycetes mostly reside in the oral cavity, upper respiratory tract, digestive tract, and urogenital tract in humans and animals, as part of the normal flora. Actinomycetes are recognized to be a cause of chronic appendicitis. Actinomycetes are also associated with Crohn-like appendicitis with significant fibrosis, transmural inflammation, lymphoid hyperplasia, and granuloma[29]. TM7x is a saccharifying bacteria, which is a parasitic bacterium that interacts with core members of Actinomycetes. A previous study suggested that ultra-small bacteria may have the ability to regulate the immune response of normal hosts, and there was a signal overlap between TM7x and basibiont Actinomyces odontolyticus species (XH001) by metabolic pathway prediction[30]. Moreover, through *in vitro* experiments, the authors demonstrated that TM7x inhibited TNF-α expression in XH001-induced macrophages[31]. Consequently, the interaction between Actinomycetes and TM7x may promote terminal ileal NLH.

In this study, we found that the abundance of Fusobacterium nucleatum increased in the test group, and this increase is possibly related to terminal ileal NLH. Fusobacterium nucleatum has been reported to be enriched in colorectal cancer tissues and played a crucial role in the occurrence and development of colorectal cancer[32]. It can adhere to and invade intestinal epithelial cells and activate the β-catenin pathway by releasing FadA adhesin and binding with cadherin E-cadherin, thus promoting inflammation and tumor response[33]. Recently, it has been reported that Fusobacterium nucleatum macromolecules (> 50 KDA) have a proinflammatory effect on human intestinal epithelium, and the outer membrane vesicles can promote the secretion of proinflammatory cytokines, including IL-8 and TNFα by epithelial cells[34]. Further animal experiments also verified the proinflammatory effect of Fusobacterium nucleatum on intestinal epithelium. Thus, Fusobacterium nucleatum may be associated with the development of terminal NLH.

In our study, the abundance of Peptostreptococcus increased in the test group. Anaerobic peptostreptococcus and Peptostreptococcus magnus are most commonly in the genus of Peptostreptococcus, among which Anaerobic peptostreptococcus is the most common pathogen. Anaerobic peptostreptococcus is a gram-positive anaerobic bacteria commonly residing in the oral cavity and digestive tract. The abundance of Anaerobic peptostreptococcus in the stool samples of patients with colorectal cancer was reported to be higher than healthy volunteers. In vitro studies have shown that Anaerobic digestion streptococcus interacts with TLR2 and TLR4 in colon cells and increases the level of active oxides, thereby promoting cholesterol synthesis and cell proliferation[35]. The PCWBR2 integrin α2/β1-PI3K-Akt-NF-κB signal axis has been reported to be involved in the development of colorectal cancer[36]. Peptostreptococcus may be related to terminal ileal inflammation and NLH, although further confirmation of this is needed in the future.

Metabolic pathways, such as the Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis, increased in the test group in the current study. Peptidoglycan, a bacterial cell wall component, is a conserved pathogen-associated molecule that is involved in the innate immune system because it recognizes pattern recognition receptors that are secreted and expressed in or on the cell surface[37]. Transfer RNAs (tRNAs) mainly participates in protein translation by transporting amino acids to the ribosome. Nevertheless, accumulating evidence has shown that tRNAs are closely associated with

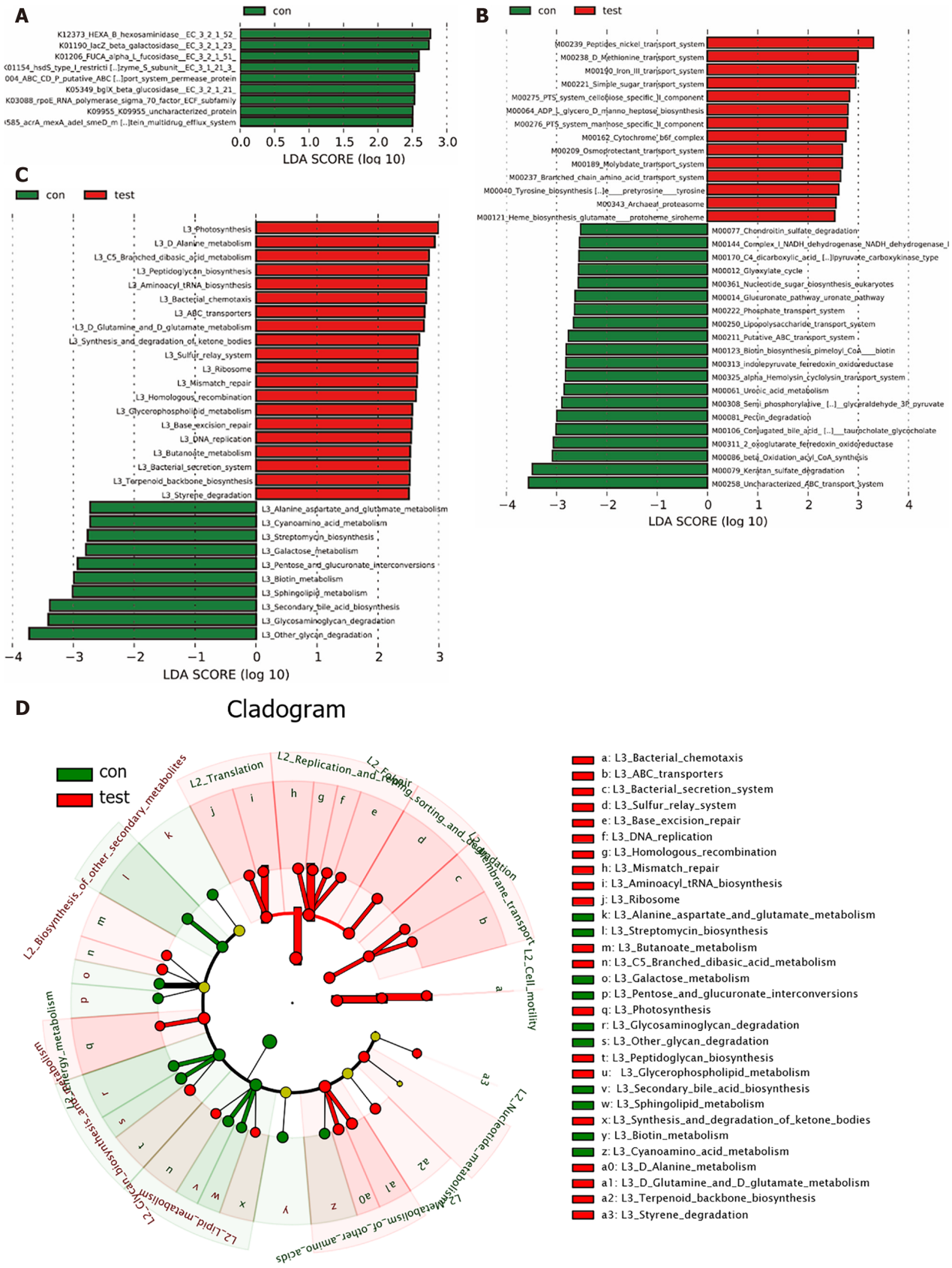


Figure 5 Predictions of the functional alteration to the gut microbiomes in patients with nodular lymphoid hyperplasia in the terminal ileum. A: Decreased the yoto Encyclopedia of Genes and Genomes orthologous groups in patients with terminal ileal nodular lymphoid hyperplasia (NLH) as shown by the histogram of the linear discriminant analysis (LDA) scores; B: Altered modules in patients with terminal ileal NLH (red, terminal ileal NLH tissue; green, control); C: Altered metabolic pathways in patients with terminal ileal NLH as shown by the histogram of the LDA scores (red, terminal ileal NLH tissue; green, control); D: Altered metabolic pathways in patients with terminal ileal NLH (red, terminal ileal NLH tissue; yellow, insignificant; green, control) as shown by a cladogram.

various physiological and pathological processes such as immune regulation. Aminoacyl-tRNA synthetases (ARSs) are essential components of translation in all living species, and it has taken scientists decades to confirm that eukaryotic ARSs act as global cell signaling mediators to regulate cell homeostasis beyond their intrinsic function as protein synthesis enzymes. Recent discoveries have revealed that ubiquitously expressed standby cytoplasmic ARSs sense and respond to danger signals and regulate immunity against infections, indicating their potential as therapeutic targets for infectious diseases[38,39]. Perhaps Peptidoglycan biosynthesis and Aminoacyl-tRNA biosynthesis might act as the targeted intervention sites.

The etiology of terminal ileal NLH has not been fully elucidated yet. In this study, we firstly analyzed the diversity and composition of intestinal flora in the mucosal tissues of the patients with terminal ileal NLH and predicted the metabolic pathways using 16S-rRNA technology. We subsequently found that terminal ileal NLH was related to the disturbance of the intestinal flora and certain microflora might act on the small intestinal mucosa thereby causing terminal ileal NLH. Moreover, the metabolic pathways that were predicted using PICRUSTs are possibly involved in terminal ileal NLH, which provides novel ideas for further exploration of potential molecular mechanisms. Diarrhea was frequently commonly found in patients with terminal ileal NLH, and some patients may get a satisfactory effect through probiotic supplementation. Therefore, exploring intestinal flora changes, seeking related bacteria genera in patients with terminal ileal NLH, and supplementing targeted protective bacteria or clearing targeted bacteria may reduce the risk of lymphoma and improve patient symptoms.

This study has some limitations. First, the sample size was relatively small. Although our preliminary results reveal that there was a significant difference between the two groups, studies with larger sample sizes covering different regions and populations are necessary to confirm the findings. Second, the male-female ratio in the NLH group was not balanced in this study due to the nature of terminal ileal NLH, which is thought to be significantly more common in men than women. Although the incidence of terminal ileal NLH in both men and women has not been investigated through a large-scale study, Lin *et al* observed that males outnumbered females by approximately four to one in a small-scale study[3]; their results support, to a certain degree, the suggestion that there is a higher frequence of terminal ileal NLH in males than females. We also performed a correlation analysis to compare the intestinal flora with gender using the Multivariable Association with Linear Models2, and we found that there was no correlation between the intestinal floras and gender at the phylum, genus, or OTU levels. To obtain more rigorous results, we will perform a large-scale study and ensure that there is an equal gender ratio among groups. Third, this study was only a preliminary correlation analysis between the intestinal flora and terminal ileal NLH, and no further research on the related mechanisms was performed. Further studies using animal testing *in vivo* and *in vitro* cellular experiments can be developed once our findings are verified in larger populations.

CONCLUSION

Intestinal flora disturbances are related to terminal ileal NLH, and our results show that certain microflora may act on the small intestinal mucosa and cause terminal ileal NLH. Maintaining the intestinal flora balance and supplementing targeted protective bacteria could improve terminal ileal NLH symptoms and potentially reduce the risk of lymphoma transformation.

ARTICLE HIGHLIGHTS

Research background

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the intestinal surface. NLH is linked to the immune system, and it may result from accumulation of plasma-cell precursors due to a maturational defect during the development of B lymphocytes. The intestinal microbiome plays an essential role in the immune system. However, whether the gut flora plays a role in NLH is unclear.

Research motivation

To explore the correlation between intestinal flora and terminal ileal NLH and predict the metabolic pathways that are involved in terminal ileal NLH.

Research objectives

To investigate the characteristics of the mucosal microbiata in patients with terminal ileal NLH for seeking related bacteria genera and bringing a new idea for related mechanisms.

Research methods

A total of 30 patients who underwent a colonoscopy were recruited for this study. A total of 15 Patients with terminal ileal NLH were assigned to the test group, while 15 healthy volunteers were assigned to the control group after undergoing a routine physical examination. We collected mucosal biopsy samples that were obtained *via* colonoscopy from both groups. We subsequently performed 16S-rRNA gene amplicon sequencing of these samples, and the results were evaluated using alpha diversity, beta diversity and microbial composition analyses. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used to predict the metabolic pathways and orthologous groups according to the Kyoto Encyclopedia of Genes and Genomes database.

Research results

The terminal ileal NLH group showed an increased alpha diversity.The overall intestinal microbiota in the NLH group was significantly different from that of the control group. The relative abundance of phylum Bacteroidetes was significantly lower in the NLH group, while that of Patescibacteria and Campilobacterota was significantly higher. The abundance of the genus *Bacteroides* was significantly lower in the test group. Conversely, the relative abundances of *Haemophilus, Streptococcus, Pseudomonas, Actinomyces, TM7X, Fusobacterium nucleatum, Parvimonas, Granulicatella, Helicobacter, and* the *[Eubacterium] nodatum group* were significantly higher in the test group. Metabolic pathways such as Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis were both increased in the test group.

Research conclusions

Maintaining the microbial balance and supplementing targeted protective bacteria could improve symptoms and potentially reduce the risk of lymphoma transformation in patients with terminal ileal NLH.

Research perspectives

Further research on the related mechanisms was needed to be performed in future. Further studies using animal testing *in vivo* and *in vitro* cellular experiments can be developed once our findings are verified in larger populations.

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FOOTNOTES

Author contributions: Jiang QL and Lu Y equally contributed on this manuscript, designed and conceived the experiments; Lu Y and Li WH collected patient samples and data; Jiang QL, Zhang MJ, Cui ZY and Pei ZM performed the 16S rRNA sequencing data analysis; Jiang QL and Lu YY acquired and analyzed data, wrote the manuscript; Lu YY and Wang JJ provided guidance for sample processing methodology; Lu LG reviewed the manuscript; all authors approved the final version of the article.

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Informed consent statement: The written informed consent was obtained from all the patients before sample collection.

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ORCID number: Qiao-Li Jiang 0000-0001-9352-6973; You Lu 0000-0002-4870-5785; Meng-Jie Zhang 0000-0002-6703-3777; Zhen-Yu Cui 0000-0002-2301-8065; Zhong-Mei Pei 0000-0001-6075-4961; Wen-Hua Li 0000-0001-9113-4415; Lun-Gen Lu 0000-0002-1533-4068; Jing-Jing Wang 0000-0002-6211-6717; Ying-Ying Lu 0000-0001-7646-861X.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Differential DNA methylation analysis of *SUMF2*, *ADAMTS5*, and *PXDN* provides novel insights into colorectal cancer prognosis prediction in Taiwan

Jing-Quan Su, Pin-Yu Lai, Pei-Hsuan Hu, Je-Ming Hu, Pi-Kai Chang, Chao-Yang Chen, Jia-Jheng Wu, Yu-Jyun Lin, Chien-An Sun, Tsan Yang, Chih-Hsiung Hsu, Hua-Ching Lin, Yu-Ching Chou

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Jing-Quan Su, School of Medicine, National Defense Medical Center, Taipei 114, Taiwan

Pin-Yu Lai, Pei-Hsuan Hu, Jia-Jheng Wu, Yu-Jyun Lin, Chih-Hsiung Hsu, Yu-Ching Chou, School of Public Health, National Defense Medical Center, Taipei 114, Taiwan

Je-Ming Hu, Pi-Kai Chang, Chao-Yang Chen, Division of Colorectal Surgery, Department of surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan

Je-Ming Hu, Yu-Ching Chou, Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei 114, Taiwan

Chien-An Sun, Department of Public Health, College of Medicine, Fu-Jen Catholic University, New Taipei City 242, Taiwan

Chien-An Sun, Big Data Research Center, College of Medicine, Fu-Jen Catholic University, New Taipei City 242, Taiwan

Tsan Yang, Department of Health Business Administration, Meiho University, Pingtung County 912, Taiwan

Hua-Ching Lin, Division of Colorectal Surgery, Department of Surgery, Cheng-Hsin General Hospital, Taipei 112, Taiwan

Corresponding author: Yu-Ching Chou, PhD, Professor, School of Public Health, National Defense Medical Center, No. 161 Sec. 6, Minquan E Road, Neihu District, Taipei 114, Taiwan. trishow@mail.ndmctsgh.edu.tw

Abstract

BACKGROUND

Patients with colorectal cancer (CRC) undergo surgery, as well as perioperative chemoradiation or adjuvant chemotherapy primarily based on the tumor–node–metastasis (TNM) cancer staging system. However, treatment responses and prognostic outcomes of patients within the same stage vary markedly. The potential use of novel biomarkers can improve prognostication and shared decision making before implementation into certain therapies.

AIM
To investigate whether *SUMF2*, *ADAMTS5*, and *PXDN* methylation status could be associated with CRC prognosis.

METHODS
We conducted a Taiwan region cohort study involving 208 patients with CRC recruited from Tri-Service General Hospital and applied the candidate gene approach to identify three genes involved in oncogenesis pathways. A methylation-specific polymerase chain reaction (MS-PCR) and EpiTYPER DNA methylation analysis were employed to detect methylation status and to quantify the methylation level of candidate genes in tumor tissue and adjacent normal tissue from participants. We evaluated *SUMF2*, *ADAMTS5*, and *PXDN* methylation as predictors of prognosis, including recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS), using a Cox regression model and Kaplan–Meier analysis.

RESULTS
We revealed various outcomes related to methylation and prognosis. Significantly shorter PFS and OS were associated with the CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue compared with CpG_3+CpG_7 hypomethylation [hazard ratio (HR) = 2.24, 95% confidence interval (CI) = 1.03-4.85 for PFS, HR = 2.56 and 95%CI = 1.08-6.04 for OS]. By contrast, a significantly longer RFS was associated with CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue compared with CpG_2 and CpG_13 hypomethylation [HR (95%CI) = 0.15 (0.03-0.71) for CpG_2 and 0.20 (0.04-0.97) for CpG_13]. The relationship between the methylation status of *PXDN* and the prognosis of CRC did not reach statistical significance.

CONCLUSION
Our study found that CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue was associated with significantly shorter PFS and OS compared with CpG_3+CpG_7 hypomethylation. CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue was associated with a significantly longer RFS compared with CpG_2 and CpG_13 hypomethylation. These methylation-related biomarkers which have implications for CRC prognosis prediction may aid physicians in clinical decision-making.

Key Words: DNA methylation; Biomarkers; Tumor tissue; Adjacent normal tissue; Prognosis prediction; Colorectal cancer

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Core Tip: Our research revealed that differential DNA methylation of candidate genes in tumor tissue and adjacent normal tissue can be used to evaluate colorectal cancer prognosis. Certain CpG sites and the methylation status of *SUMF2* and *ADAMTS5* were significantly associated with colorectal cancer recurrence, progression, and survival. We recommend using our findings to investigate prognostic biomarkers applicable to patients with colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths worldwide[1]. According to global estimations, in 2018, 1.8 million new cases of CRC were diagnosed, and 0.8 million people died from CRC that year[2]. By 2030, the worldwide CRC burden is predicted to increase by 60%; in 2030, it is expected that 2.2 million patients will be newly diagnosed as having CRC and 1.1 million CRC-related deaths will occur worldwide[1]. The treatment and survival of patients with CRC are closely related to cancer staging systems. The classification of CRC stages is based on the tumor– node–metastasis (TNM; T, size of the primary tumor; N, nearby affected lymph nodes; M, distant metastasis) staging system from the Eighth Edition of the

American Joint Committee on Cancer Staging Manual[3]. However, the heterogeneity of CRC means that patients with the same CRC stage may have different treatment responses and survival times[4]. Although breakthroughs in surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy have improved the survival of patients with CRC, the 5-year survival of patients with stage IV disease is as low as 14% owing to CRC's heterogeneous nature and association with diverse molecular alterations, which are involved in cancer cell progression[5]. Intensive postoperative surveillance programs are proposed after tumor resection to detect asymptomatic recurrence in advance and prolong the survival patients who may be suited to further curative therapy[6]. Therefore, identifying molecular biomarkers for predicting patient prognoses or monitoring cancer relapse is crucial.

Several studies have focused on identifying new prognostic indicators for CRC[7]. Prognostic biomarkers can be employed as personalized indicators to predict disease progression, such as early recurrence, metastasis, and mortality[8]. These biomarkers are associated with molecular patterns of genomic mutations and epigenetic alterations that lead to CRC carcinogenesis[9]. DNA methylation alterations play a pivotal role in CRC progression and metastasis[10]. Promoter DNA hypermethylation silences genes such as *MLH1*, *CDKN2A*, *MGMT*, *RUNX3*, *TPEF*, *VIM*, and *SFRP1/2/4/5*, which play crucial roles in the cell cycle, DNA repair, and signal transduction[11]. Consequently, DNA methylation of genes may be a novel epigenetic indicator of patient prognosis.

SUMF2 was regarded as one of the frequently mutated genes in CRC, and *SUMF2* mutation frequently altered pathways in the tumorigenesis of CRC[12]. *ADAMTS5* was found upregulated in CRC, which was associated with tumor progression and even unfavorable clinical outcomes[13]. To determine the effect of the DNA methylation of selected genes on CRC prognosis over 5 years, we examined methylation status and extent in tumor tissue and tumor-free areas adjacent to such tissue. We propose that the differential DNA methylation of candidate genes in tumor samples and in matched adjacent normal tissue could assist in prognosis prediction and the optimization of CRC treatment.

MATERIALS AND METHODS

Patient and specimen collection

In this retrospective cohort study, we analyzed the data of patients diagnosed as having CRC from 2006 to 2010 and who underwent surgical treatment at Tri-Service General Hospital (TSGH), Taiwan, to assess their 5-year prognosis. All participants signed informed consent forms before their involvement in this research. Then surgeons gathered specimens in patients including colorectal cancer tissue and adjacent normal regions during surgery. The tissue were deposited at -80 ℃ ultra-low temperature freezers for further analysis. The study was approved by the TSGH Institutional Review Board (TSGHIRB approval numbers 098-05-292 and 2-105-05-129). According to the clinical practice guidelines of the Division of Colon and Rectal Surgery of TSGH, patients with CRC should return to the outpatient department for a follow-up every 3 mo in the first year after surgery and once every 3 to 6 mo afterward. The clinical and demographic characteristics of enrolled patients, including sex, age at surgery, clinical staging, tumor size, histological grade, lymph node count, tumor location, and adjuvant chemotherapy as well as follow-up information on recurrence, metastasis, and survival were acquired from the cancer registration database of TSGH.

Recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were calculated from the date of surgery to disease progression (inclusive of cancer recurrence or metastasis), death from any cause, or until the final follow-up date before December 31, 2010. In total, 208 patients who met the inclusion criteria were enrolled. A flow diagram of the study's design is presented in Figure 1.

Gene selection and DNA extraction

The candidate gene approach was applied for genetic association studies, which has been widely used to investigate novel prognostic biomarkers related to CRC[14]. First, we searched for genes whose expression might influence CRC prognosis by browsing PubMed (https://pubmed.ncbi.nlm.nih.gov/), University of California, Santa Cruz Genome Browser (https://genome.ucsc.edu/) and Prediction of Clinical Outcomes from Genomic Profiles (https://precog.stanford.edu/). Subsequently, we confirmed gene methylation differences in CRC tissue and normal tissue using the Shiny Methylation Analysis Resource Tool website (http://bioinfo-zs.com/smartapp/). We then searched PubMed (https://pubmed.ncbi.nlm.nih.gov/) to review related literature with the keyword "gene name + colorectal cancer". If the number of results was less than 30, it was thought that the gene had been rarely studied for colorectal cancer. Those genes which met the above three conditions were included in this study. Therefore, we chose 16 candidate genes include *CFLAR*, *RBM44*, *ABCG1*, *WDR74*, *ZNF292*, *EFHA2*, *PXDN*, *TEC*, *CDH2*, *ADAMTS5*, *COL4A2*, *PCGF2*, *EMID2*, *GRPEL2*, *DKK2* and *SUMF2*. Because of the limited resources and experimental results, we narrowed down to *SUMF2*, *ADAMTS5*, and *PXDN*. These three candidate genes involved in the pathways associated with cancer stages and prognosis, such as inflammation, epithelial–mesenchymal transition, tumor migration, and angiogenesis.

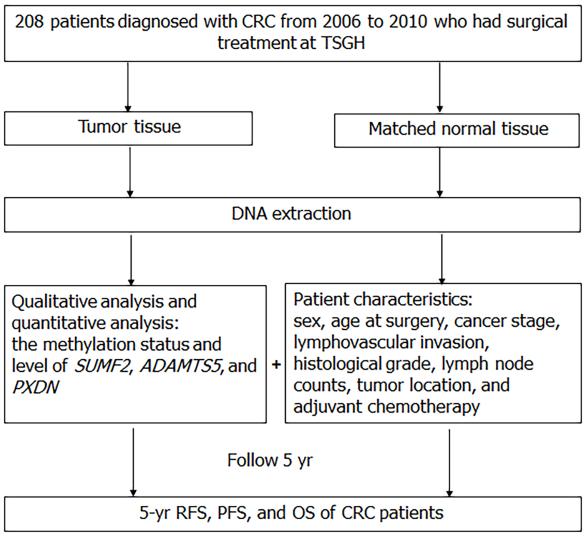


Figure 1 The study design flow-diagram. CRC: Colorectal cancer; TSGH: Tri-Service General Hospital; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5; *PXDN*: Peroxidasin; RFS: Recurrence-free survival; PFS: Progression-free survival; OS: Overall survival.

In accordance with the manufacturer's instructions, cellulose-coated magnetic beads were used to extract genomic DNA from the samples by using the Genomic DNA Tissue Kit (Catalog No. 69504; Qiagen, Taipei, Taiwan). We used sterile blades to cut about 10 mg tissue minced into the 1.5 mL microcentrifuge tube, and 200 μL Lysis Buffer and 6 μL Proteinase K were prepared and added. The 1.5 mL microcentrifuge tube was placed at 56°C water bath machine for 8-10 h after orbital shaker and centrifuge use. We confirmed that tissues were dissolved as clarified liquid, and then the tissue would have a vortex and centrifugation. Next, we used pipette to put tissue lysate into the 96 Deep Well Plate of the MagCore Compact Automated Nucleic Acid Extractor (Catalog No. MCA0801; RBC Bioscience, Taipei, Taiwan). Subsequently, we used the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, United States) to modify isolated DNA using sodium bisulfite.

Methylation-specific polymerase chain reaction and EpiTYPER DNA methylation analysis

The gene methylation statuses of *SUMF2*, *ADAMTS5*, and *PXDN* were evaluated through a methylation-specific polymerase chain reaction (MS-PCR). The total volume of the reaction solution was 20 μL, and it contained HotStart Taq Premix (RBC Bioscience, Taipei, Taiwan) (9 μL for *SUMF2* and *PXDN*; 10 μL for *ADAMTS5*), 0.5 μL of forward and reverse primers, 1 μL of bisulfite-modified DNA, and pure water (9 μL for *SUMF2* and *PXDN*; 8 μL for *ADAMTS5*).

For MS-PCR, the oligonucleotide primers, annealing temperature of each primer used for amplification, and PCR product sizes were described in Table 1. PCR cycling was performed as follows: 10 min at 95 °C, 38 cycles of denaturation for 30 s at 95 °C, 30-s annealing at a gene-appropriate temperature, 30-s elongation at 72 °C, final extension for 7 min at 72 °C, and holding at 4 °C. After amplification, PCR products were mixed with a loading buffer, electrophoresed (100 V for 28–30 min) on 2.75%-4% agarose gels using 1–2 μL of gel-stained dye, and visualized using an ultraviolet transilluminator. To confirm our experiment results were without error, we used SssI-treated DNA as positive control, and sterile water as negative control. Figure 2 showed that the methylation-specific polymerase chain reaction (MS-PCR) results of *PXDN* gene in CRC patients. Negative control, positive control and sterile water represent unmethylation-specific reaction, methylation-specific reaction and no contaminant reaction for PCR, respectively.

We further identified the CpG sites of *SUMF2* and *ADAMTS5* (Figure 3) and analyzed DNA methylation changes by using an Agena Bioscience MassARRAY system with EpiTYPER biochemistry (Agena Bioscience, San Diego, CA), an advanced method for quantitative DNA analysis.

The oligonucleotide primers, annealing temperature of each primer used for amplification, and PCR product sizes used in the EpiTyper assay were described in Table 1. The PCR cycling protocols were as follows: 15 min at 95 °C, 38 cycles of denaturation for 30 s at 95 °C, 30-s annealing at a gene-appropriate temperature, 30-s elongation at 72 °C, final extension for 10 min at 72 °C, and holding at 4 °C. In vitro transcription and base-specific cleavage were performed using the MassCLEAVE kit (Agena Bioscience). In total, 0.22 μL of T cleavage mix, 3.14 mmol/L DTT, 20 U of T7 RNA and DNA polymerase, and 0.09 mg/mL RNase A were prepared and added to a 7 μL reaction solution with shrimp alkaline phosphatase inactive PCR product. The final product was stored at 37 °C for 3 h. After the addition of a cation exchange resin to remove salt that remained after the reactions, the EpiTYPER reaction products were loaded onto the matrix pad of a SpectroCHIP Array (Agena Bioscience). The size

Table 1 Primer sequences, annealing temperature and product size for MS-PCR and EpiTYPER DNA methylation analysis of target genes

Genes		Forward primer (5' → 3')	Annealing temperature (°C)	Product size (bp)
SUMF2	M	F:TTTGATTATGGTCGGTTTTGC	59.4	191
		R:GACTACTTACAACTCCCCTAACGAC		
	U	F:TTTTTTGATTATGGTTGGTTTTGTG	60.6	198
		R:CCCAACTACTTACAACTCCCCTAACA		
	Q	F:TTTGTTATAGAGGGATGGGAGATAG aggaagagag	60	232
		R:CAAAATAAACAACACTCCAAATTCA cagtaatacgactcactatagggagaaggct		
ADAMTS5	M	F:GTTATTGTCGTGGAGCGTTAGC	59.4	170
		R:CCTACCTCCCGTACTTCCCG		
	U	F:TTATTGTTGTGGAGTGTTAGTGTTT	59.4	169
		R:CCTACCTCCCATACTTCCCACAT		
	Q	F:aggaagagagTTGAAATTGTTATTGTAGGATGGTATG	61.3	245
		R:cagtaatacgactcactatagggagaaggctAATTAAAACAAAAATACAAAAAAACAACC		
PXDN	M	F:TATGCGGGACGAGAACGAGA	61.6	137
		R:ACTTAAACAACTCCGTAACAATACGAT		
	U	F:GTGTATGTGGGATGAGAATGAGAG	60.4	142
		R:CAACTTAAACAACTCCATAACAATACAA		

MS-PCR: Methylation-specific polymerase chain reaction; SUMF2: Sulfatase modifying factor 2; ADAMTS5: ADAM metallopeptidase with thrombospondin type 1 motif 5; PXDN: Peroxidasin; M: Methylation; U: Unmethylation; Q: Quantitative analysis.

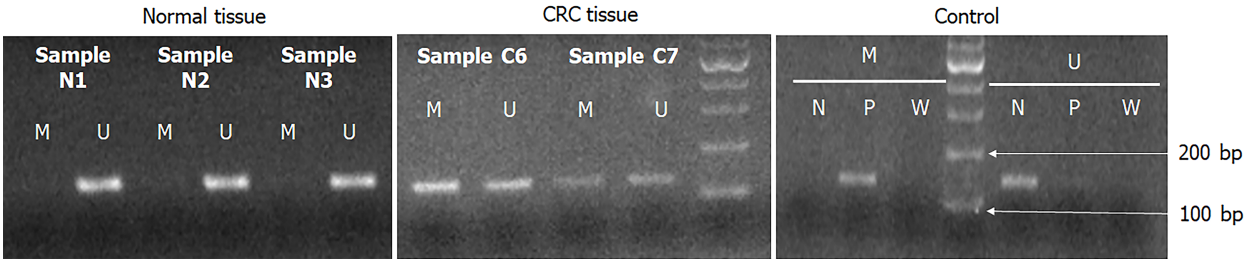


Figure 2 The methylation-specific polymerase chain reaction results of *PXDN* gene in colorectal cancer patients. Negative control, positive control and sterile water represent unmethylation-specific reaction, methylation-specific reaction and no contaminant reaction for polymerase chain reaction, respectively. CRC: Colorectal cancer.

and mass of the resulting cleavage products varied based on sequence changes. The fragment contained CpG sites had two kinds of molecular weight (GC or AC) because of the methylation status. There are 16 Daltons of signal divergence between the methylated and unmethylated sequence, which was observed by the mass spectrometry. These differences provided quantitative information on each target fragment, which was acquired using the MassARRAY Analyzer 4 (Agena Bioscience). Signal shifts of fragments indicated methylation events at single CpGs or small groups of CpGs (CpG units), and signal intensity was linked to DNA methylation extent, which was analyzed using EpiTYPER software (Agena Bioscience).

Statistical analysis

For each candidate gene, different DNA methylation statuses related to RFS, PFS, and OS were investigated using univariate Cox proportional hazards analyses. The resultant multivariate Cox proportional hazards regression model was then used to verify the independent prognostic effects of gene methylation status after adjustment for various clinical variables (sex, age at surgery, stage, and lymph node counts). The 5-year RFS, PFS, and OS curves for the methylation status of the selected genes were presented using a Kaplan–Meier survival analysis and compared using a log-rank test. We used SPSS

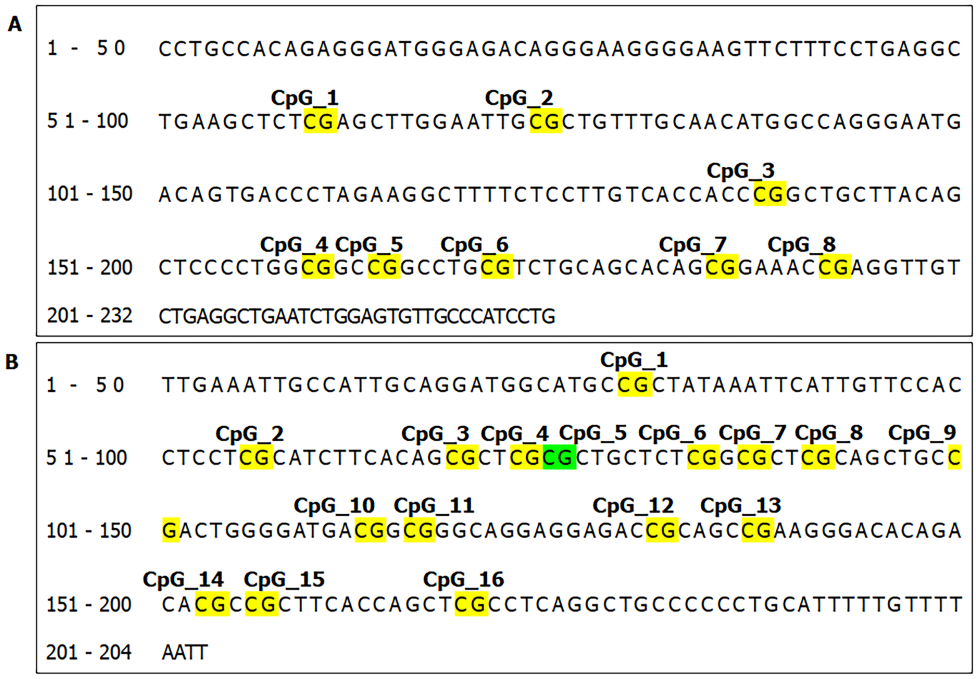


Figure 3 The location of informative CpG sites in the (A) *SUMF2* and (B) *ADAMTS5* promoter region.

version 23 (IBM SPSS Statistics 23) for statistical analyses. All *P* values were calculated from two - sided tests, and *P* value below 0.05 represented statistical significance.

RESULTS

Patient characteristics

In total, 208 patients diagnosed as having CRC at TSGH were recruited to participate. We analyzed tumor samples and adjacent nontumorous tissue of enrollees. The correlation of the candidate genes' methylation status and extent with the demographic and clinicopathological characteristics of patients was assessed, and the results are presented in Table 2. Normal tissue with *SUMF2* methylation significantly belonged to poor or undifferentiated histological grade. Tumor tissue with *PXDN* methylation significantly belonged to lower stages and had less lymphovascular invasion. No other associations were found between the methylation statuses of these three genes and the demographic or clinicopathological variables of the participants.

Quantitative analysis of genes

Although the selected genes were methylated in both tumor tissue and matched normal tissue (*SUMF2*, 50% *vs* 52.9%; *ADAMTS5*, 75.0% *vs* 62.9%; *PXDN*, 78.2% *vs* 72.4%), we observed that the differential DNA methylation of candidate genes, especially *SUMF2* and *ADAMTS5*, tended to affect patient prognosis according to the Kaplan–Meier method. To further investigate the extent and patterns of gene methylation in patients with CRC, we analyzed *SUMF2* and *ADAMTS5* methylation levels in primary CRC tissue samples and in adjacent normal samples. A higher percentage of *SUMF2* genes methylation at loci CpG_1, CpG_2, CpG_3 and CpG_7 was detected in primary CRC samples compared with adjacent normal tissue. We also found a higher level of *ADAMTS5* genes methylation at loci CpG_1, CpG_2, CpG_9, CpG_10.11, CpG_12, CpG_13, CpG_14.15, and CpG_16 in CRC tissue than in adjacent normal samples. Both findings reached statistical significance (Table 3).

Relationship between gene methylation and prognosis

The correlation between the methylation status of each gene and the 5-year RFS, PFS, and OS of patients with CRC was analyzed. We did not observe a significant correlation of the *SUMF2*, *ADAMTS5*, and *PXDN* methylation statuses of normal and tumor tissue and with patients' 5-year RFS, PFS, and OS in univariate and multivariate Cox proportional hazards regression analyses. The methylation of *SUMF2* in normal tissue was associated with poorer 5-year PFS and OS in a Kaplan–Meier survival analyses (*P* = 0.500 and 0.260, respectively). In a univariate analysis, we observed that patients with a stage III+IV disease had a poor RFS, PFS, and OS [hazard ratio (HR) = 4.68 and 95% confidence interval (95%CI) = 2.09-10.46 for RFS, 2.21 (1.25-3.91) for PFS, and 1.90 (1.00-3.61) for OS], even after adjustment for

Table 2 Characteristics and distribution of methylation status in patients with colorectal cancer (*n* = 208)

Characteristics	Total	Methylation status					
		SUMF2		*ADAMTS5*		*PXDN*	
		Normal	Tumor	Normal	Tumor	Normal	Tumor
Sex							
Male	103 (49.5)	20 (60.6)	27 (55.1)	28 (60.9)	44 (77.2)	35 (76.1)	44 (77.2)
Female	105 (50.5)	17 (45.9)	25 (45.5)	38 (64.4)	49 (73.1)	41 (69.5)	53 (79.1)
χ^2 (*P* value)		0.97 (0.324)	0.62 (0.432)	0.03 (0.866)	0.1 (0.755)	0.28 (0.596)	< 0.01 (0.969)
Age at surgery							
mean ± SD	64.3 ± 14.6	64.9 ± 14.2	66.9 ± 15.8	66.4 ± 14.8	67.7 ± 15.5	65.0 ± 14.4	66.2 ± 15.0
< 65	103 (49.5)	17 (51.5)	26 (53.1)	28 (58.3)	43 (72.9)	34 (70.8)	46 (78.0)
≥ 65	105 (50.5)	20 (54.1)	26 (47.3)	38 (66.7)	50 (76.9)	42 (73.7)	51 (78.5)
χ^2 (*P* value)		< 0.01 (1.00)	0.15 (0.694)	0.46 (0.498)	0.1 (0.755)	0.01 (0.915)	< 0.01(1.00)
Stage							
I	29 (13.9)	7 (58.3)	7 (50.0)	9 (50.0)	12 (63.2)	12 (66.7)	17 (89.5)
II	77 (37.0)	13 (48.1)	17 (45.9)	21 (56.8)	33 (75.0)	29 (78.4)	39 (88.6)
III	68 (32.7)	13 (65.0)	21 (63.6)	22 (68.8)	31 (75.6)	22 (68.8)	27 (65.9)
IV	34 (16.3)	4 (36.4)	7 (35.0)	14 (77.8)	17 (85.0)	13 (72.2)	14 (70.0)
χ^2 (*P* value)		2.77 (0.429)	4.5 (0.212)	4.06 (0.255)	2.5 (0.476)	1.17 (0.760)	8.69[a] (0.034)
5-yr recurrence[1]							
No	141 (82.0)	28 (51.9)	35 (49.3)	43 (56.6)	61 (70.9)	54 (71.1)	71 (82.6)
Yes	31 (18.0)	4 (40.0)	9 (60.0)	12 (80.0)	14 (82.4)	11 (73.3)	12 (70.6)
χ^2 (*P* value)		0.12 (0.731)	0.22 (0.639)	1.98 (0.160)	0.45 (0.504)	< 0.01 (1.00)	0.65 (0.421)
5-yr all-cause death							
No	168 (80.8)	29 (54.7)	43 (51.8)	50 (61.0)	79 (78.2)	59 (72.0)	77 (76.2)
Yes	40 (19.2)	8 (47.1)	9 (42.9)	16 (69.6)	14 (60.9)	17 (73.9)	20 (87.0)
χ^2 (*P* value)		0.07 (0.786)	0.24 (0.625)	0.26 (0.611)	2.15 (0.142)	< 0.01 (1.00)	0.71 (0.399)
5-yr progression							
No	155 (74.5)	28 (56.0)	39 (50.0)	45 (58.4)	73 (76.8)	56 (72.7)	74 (77.9)
Yes	53 (25.5)	9 (45.0)	13 (50.0)	21 (75.0)	20 (69.0)	20 (71.4)	23 (79.3)
χ^2 (*P* value)		0.32 (0.570)	< 0.01(1.00)	1.75 (0.185)	0.38 (0.540)	< 0.01 (1.00)	< 0.01 (1.00)
Lymphovascular invasion[1]							
No	106 (52.5)	20 (48.8)	25 (48.1)	32 (56.1)	46 (73.0)	41 (71.9)	55 (87.3)
Yes	96 (47.5)	16 (57.1)	27 (51.9)	34 (72.3)	47 (78.3)	34 (72.3)	41 (68.3)
χ^2 (*P* value)		0.19 (0.662)	0.04 (0.845)	2.26 (0.133)	0.23 (0.634)	< 0.01 (1.00)	5.44[a] (0.02)
Histological grade[1]							
Well or moderately	156 (89.7)	27 (48.2)	35 (47.3)	52 (64.2)	67 (74.4)	58 (71.6)	67 (74.4)
Poor or undifferentiated	18 (10.3)	6 (100.0)	9 (64.3)	7 (70.0)	14 (82.4)	9 (90.0)	13 (76.5)
χ^2 (*P* value)		3.94[a] (0.047)	0.76 (0.382)	< 0.01 (0.99)	0.15 (0.697)	0.75 (0.387)	< 0.01 (1.00)
Lymph node counts[1]							
0-11	34 (18.4)	7 (63.6)	7 (50.0)	12 (80.0)	15 (83.3)	13 (86.7)	16 (88.9)
≥ 12	151 (81.6)	29 (52.7)	40 (50.0)	50 (61.7)	70 (73.7)	58 (71.6)	70 (73.7)
χ^2 (*P* value)		0.07 (0.786)	< 0.01 (1.00)	1.14 (0.287)	0.33 (0.568)	0.81 (0.368)	1.18 (0.278)

Tumor location[1]							
Colon	147 (79.9)	28 (50.0)	38 (50.7)	53 (67.9)	62 (70.5)	60 (76.9)	68 (77.3)
Rectum	37 (20.1)	8 (80.0)	9 (47.4)	9 (50.0)	23 (92.0)	11 (61.1)	18 (72.0)
χ^2 (*P* value)		1.99 (0.158)	< 0.01(1.00)	1.35 (0.245)	3.76 (0.052)	1.17 (0.280)	0.08 (0.780)
Adjuvant chemotherapy[1]							
No	54 (29.3)	10 (58.8)	12 (42.9)	15 (55.6)	25 (73.5)	20 (74.1)	29 (85.3)
Yes	130 (70.7)	26 (53.1)	35 (53.0)	47 (68.1)	60 (75.9)	51 (73.9)	57 (72.2)
χ^2 (*P* value)		0.02 (0.898)	0.46 (0.499)	0.85 (0.358)	0.01 (0.971)	< 0.01(1.00)	1.59 (0.207)

[1]The total number of colorectal cancer patients does not correspond because of missing data.
[a]*P* < 0.05. *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5; *PXDN*: Peroxidasin; SD: Standard deviation.

Table 3 Methylation level of sulfatase modifying factor 2 and ADAM metallopeptidase with thrombospondin type 1 motif 5 in normal tissue and tumor tissue (*n*= 208)

	Normal			Tumor			*P* value
	n[1]	Median	mean ± SD[2]	*n*[1]	Median	mean ± SD[2]	
SUMF2							
CpG_1	69	0.40	0.43 ± 0.15	104	0.53	0.55 ± 0.17	< 0.001
CpG_2	70	0.56	0.56 ± 0.11	104	0.76	0.73 ± 0.13	< 0.001
CpG_3	70	0.38	0.39 ± 0.11	104	0.54	0.54 ± 0.17	< 0.001
CpG_7	48	0.64	0.64 ± 0.15	80	0.87	0.81 ± 0.20	0.001
ADAMTS5							
CpG_1	66	0.06	0.08 ± 0.07	95	0.19	0.25 ± 0.20	< 0.001
CpG_2	70	0.06	0.08 ± 0.06	105	0.20	0.24 ± 0.18	< 0.001
CpG_9	69	0.06	0.07 ± 0.06	91	0.21	0.25 ± 0.18	< 0.001
CpG_10.11	69	0.08	0.10 ± 0.09	103	0.30	0.34 ± 0.22	< 0.001
CpG_12	65	0.09	0.10 ± 0.08	98	0.19	0.24 ± 0.21	0.001
CpG_13	63	0.07	0.12 ± 0.14	94	0.15	0.22 ± 0.22	0.009
CpG_14.15	71	0.32	0.33 ± 0.05	105	0.43	0.44 ± 0.11	< 0.001
CpG_16	71	0.10	0.11 ± 0.04	105	0.19	0.22 ± 0.12	< 0.001

[1]The total number of colorectal cancer patients does not correspond because of missing data.
[2]Represent the ratio of DNA methylation.
SD: Standard deviation; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5.

confounding factors in the multivariable analysis of tumor tissue [HR (95%CI) = 9.85 (2.20-44.15) for RFS, 4.08 (1.49-11.14) for PFS, and 4.70 (1.50-14.74) for OS]. Methylation of *ADAMTS5* in normal or tumor tissue was associated with a poor 5-year RFS (*P* = 0.144 for normal tissue and 0.332 for tumor tissue), and *ADAMTS5* methylation in normal tissue was associated with a poor 5-year PFS (*P* = 0.176). Patients with a stage III+IV disease had a poor RFS, PFS, and OS even after adjustment for confounding factors in the multivariable analysis [HR (95%CI) = 4.82 (1.33-17.48) for RFS (normal tissue), 7.37 (2.11-25.83) for RFS (tumor tissue), 2.83 (1.19-6.74) for PFS (tumor tissue), and 2.63 (1.02-6.81) for OS (tumor tissue)]. Finally, no relationship was observed between *PXDN* methylation status and CRC prognosis.

We assessed the associations of *SUMF2* and *ADAMTS5* with prognosis using a quantitative analysis (Table 4). To enhance statistical power, we classified the methylation level of candidate genes into hypermethylation and hypomethylation groups according to the median value shown in Table 3, which functioned as the threshold value. On the basis of consecutive 5-year RFS, PFS, and OS Kaplan–Meier plot validation, a clear trend of differences was observed between hypermethylation and hypomethylation of the *SUMF2* gene at CpG_3 and CpG_7 Loci from tumor tissue. We further focused

Table 4 Multivariate 5-year progression and survival analysis of *SUMF2* and *ADAMTS5* gene

	RFS		PFS		OS	
	cHR (95%CI)	aHR (95%CI)[1]	cHR (95%CI)	aHR (95%CI)[1]	cHR (95%CI)	aHR (95%CI)[2]
SUMF2 in tumor tissue						
CpG_3+CpG_7						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	2.37 (0.86-6.55)	1.64 (0.55-4.89)	2.24 (1.03-4.85)[a]	2.05 (0.91-4.62)	2.56 (1.08-6.04)[a]	3.53 (1.35-9.26)[a]
ADAMTS5 in normal tissue						
CpG_2						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	0.15 (0.03-0.71)[a]	0.17 (0.03-0.95)[a]	0.57 (0.24-1.37)	0.54 (0.21-1.41)	0.82 (0.31-2.16)	0.94 (0.31-2.85)
CpG_13						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	0.20 (0.04-0.97)[a]	0.16 (0.03-0.85)[a]	0.48 (0.19-1.19)	0.45 (0.17-1.18)	0.50 (0.19-1.30)	0.72 (0.24-2.15)

[1]Adjusted for sex, age, and stage.

[2]Adjusted for sex, age, stage, and lymph node counts.

[a]$P < 0.05$. RFS: Recurrence-free survival; PFS: Progression-free survival; OS: Overall survival; cHR: Crude hazard ratio; aHR: Adjusted hazard ratio; CI: Confidence interval; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5.

on the methylation statuses at CpG_3+CpG_7 to evaluate their relationship with patient prognosis. The 5-year PFS and OS curves revealed a significant difference between the hypermethylation and hypomethylation of the *SUMF2* gene at CpG_3+CpG_7 Loci from tumor tissue ($P = 0.026$ for PFS and 0.036 for OS; Figure 4). Compared with the CpG_3+CpG_7 hypomethylation of tumor tissue, a significantly shorter PFS and OS were observed for CpG_3+CpG_7 hypermethylation [HR (95%CI) = 2.24 (1.03-4.85) for PFS and 2.56 (1.08-6.04) for OS]. After adjustment for confounders in the multivariable analysis, the shorter OS associated with CpG_3+CpG_7 hypermethylation remained significant [HR (95%CI) = 3.53 (1.35-9.26)], whereas the shorter PFS associated with CpG_3+CpG_7 hypermethylation did not [HR (95%CI) = 2.05 (0.91-4.62)] (Table 4).

The 5-year RFS curves showed a significant difference between hypermethylation and hypomethylation of the *ADAMTS5* gene at CpG_2 and CpG_13 from normal tissue ($P = 0.006$ for CpG_2 and 0.026 for CpG_13; Figure 5). Compared with CpG_2 and CpG_13 hypomethylation of normal tissue, a significantly longer RFS was observed for CpG_2 and CpG_13 hypermethylation [HR (95%CI) = 0.15 (0.03-0.71) for CpG_2 and 0.20 (0.04-0.97) for CpG_13]. After adjustment for confounders in the multivariable analysis, the longer RFS associated with CpG_2 and CpG_13 hypermethylation remained significant [HR (95%CI) = 0.17 (0.03-0.95) for CpG_2 and 0.16 (0.03-0.85) for CpG_13] (Table 4).

DISCUSSION

The TNM staging system based on tumor depth, nodal status, and metastasis guides treatment strategies and improves the accuracy of predicting prognosis. However, the etiologically heterogeneous characteristics of CRC contributes to differences in survival between patients with the same TNM stage of CRC. Because of this heterogeneity, TNM staging of CRC requires further classification for better disease management. Therefore, combining other classifications related to several biologic mechanisms with TNM staging is required[15].

Aberrant DNA methylation of certain loci is involved in the aberrant expression of oncogenes through the hypomethylation of CpG islands in promoters. On the other hand, transcriptional silencing by the hypermethylation of CpG islands in promoters is observed in tumor-suppressor genes[16]. Aberrant DNA methylation elicits apoptosis[17], metastasis[18], cell adherence[19], tumor progression [18,20], and resistance to current anticancer therapies[21]. Hypermethylation at CpG islands of genes has promise as a robust and valid diagnostic method for CRC[22]. Additionally, methylation-based molecular markers can be employed as prognostic or predictive biomarkers for CRC to improve clinical management; such markers can predict malignant tumor potential and survival outcomes[23].

Globally acquired DNA hypomethylation profiles represent a key step in the development and advancement of CRC because hypomethylation triggers genomic instability and the global loss of imprinting[24]. This epigenetic change contributes to the activation of oncogenes and mainly affects

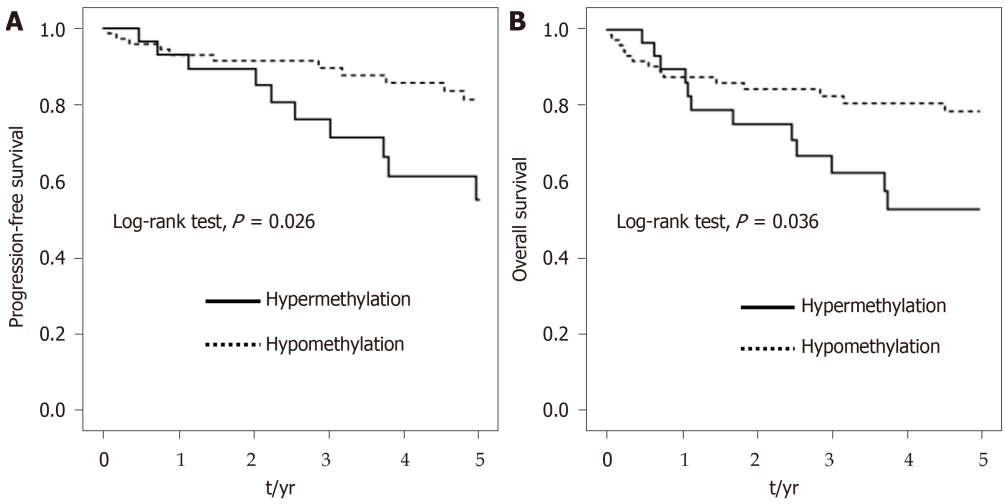


Figure 4 Kaplan–Meier survival curves depicting the effect of hypermethylation and hypomethylation of *SUMF2* at CpG_3+CpG_7 from tumor tissue on 5-year (A) progression-free survival and (B) overall survival of colorectal cancer patients.

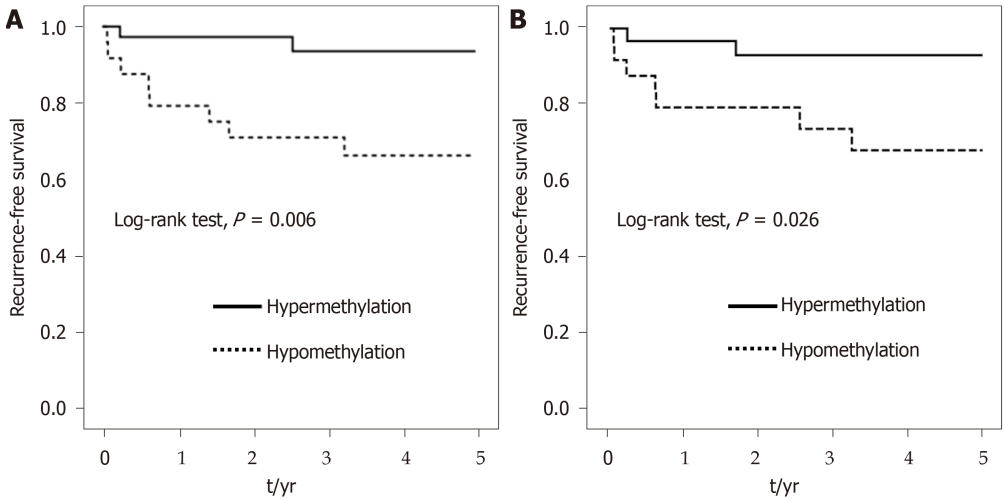


Figure 5 Kaplan–Meier survival curves depicting the effect of hypermethylation and hypomethylation of *ADAMTS5* at (A) CpG_2 and (B) CpG_13 from normal tissue on 5-year recurrence-free survival of colorectal cancer patients.

repetitive transposable elements, such as long interspersed nuclear element-1 (LINE-1 or L1), which represent 17% of the human genome[25]. LINE-1 is more hypomethylated in CRC tumors compared with adjacent normal tissue, and this is linked to metastasis. Moreover, LINE-1 is more hypermethylated in the neoplastic tissue of patients treated for CRC compared with untreated patients, and after neoadjuvant treatment, poor survival was observed in patients with tumor LINE-1 hypomethylation[26]. The results of a meta-analysis also support the idea that LINE-1 hypomethylation is considerably associated with the shortened OS and disease-free survival (DFS) of patients with CRC [27].

In this study, we analyzed 208 of each tumor tissue samples and normal-appearing tissue samples from patients with CRC. CpG_3+CpG_7 hypermethylation of *SUMF2* in tumor tissue was strongly related to poorer 5-year PFS and OS according to a Cox proportional hazards regression model and Kaplan–Meier curves. By contrast, CpG_2 and CpG_13 hypermethylation of *ADAMTS5* in adjacent normal tissue was significantly associated with better 5-year RFS than CpG_2 and CpG_13 hypomethylation of *ADAMTS5* in adjacent normal tissue. Finally, no correlation was observed between the promoter methylation status of *PXDN* and patient prognosis regardless of the tissue type analyzed.

Therefore, our findings indicate that *SUMF2* could be a tumor-suppressor gene, whereas *ADAMTS5* may be classified as a CRC oncogene. These findings can be applied along with current staging to modify the treatment of patients with CRC, and the genes discussed identified herein can serve as appropriate biomarkers to identify patients at a higher risk of having a poor prognosis and to indicate requirements for intensive follow-up.

We revealed that *ADAMTS5* methylation status in tumor-free areas adjacent to tumors was significantly associated with the prognosis of patients with CRC. The progressive accumulation of genetic mutations and DNA methylation changes in normal tissue around tumors lead to the development and progression of adenomas, which might become adenocarcinomas. Such alterations are a result of field cancerization[28]. Studies have demonstrated that the aberrant methylation of cancer-related genes could serve as epigenetic markers for CRC risk owing to the field of susceptibility[29], and such findings are consistent with our finding that compared with abnormal DNA methylation in tumor tissue, abnormal DNA methylation in adjacent normal tissue is highly correlated with an unfavorable prognosis after surgical resection.

SUMF2, located in the luminal space of the endoplasmic reticulum, is a member of the formylglycine-generating enzyme family and regulates the activity of sulfatase and the formation of formylglycine [30]. According to a whole-genome microarray expression study, Ala62Thr changes in ZNF365 isoform D are related to the altered expression of *SUMF2* in patients with Crohn disease[31]. According to Liang *et al*[32], *SUMF2* was one of the main genes with significant mutation in CRC. No report has examined the association between *SUMF2* methylation status and the prognosis of patients with CRC. *ADAMTS* family members containing disintegrin domains, metalloproteinases, and thrombospondin motifs likely contribute to malignant transformation such as cancer cell adhesion, fusion, migration, proliferation, and metastasis in CRC through their modification of the structure and function of the extracellular matrix (ECM)[33] and desmoplastic reactions (the overgrowth of fibrous connective tissue around carcinoma cells)[34]. The expression level of *ADAMTS5* was increased in late CRC stages, and *ADAMTS5* may have served as the fundamental component of tumor invasion through degrading ECM so as to promote tumor progression to more advanced stages of CRC[35]. The ratios of lymphatic invasion and lymph node metastasis were significantly higher in patients with CRC and high *ADAMTS5* expression, but such expression did not affect OS and DFS[36]. However, Li *et al*[37] suggested that *ADAMTS5* overexpression inhibited the invasion and migration of CRC, and *ADAMTS5* was more hypermethylated in tumor tissue compared with normal tissue, corresponding to poor OS and DFS. This result was not observed in our research. *PXDN*, which regulates cell plasticity and remodels the ECM by encoding ECM protein with peroxidase activity[38], engages in epithelial mesenchymal transition(EMT)[39]. EMT is a process by which epithelial cells lose cell polarity and cell–cell adhesion and acquire migratory and invasive capabilities to become mesenchymal cells; this activity has shown to occur during the initiation of metastasis[40]. A few studies have studied *PXDN* in the context of cancer; for example, the increased expression of *PXDN* has been shown in patients with melanoma[41] as well as in patients with brain[42], breast[43], ovarian[44] or prostate[45] cancer. No reports have examined the correlation between *PXDN* expression and the prognosis of patients with CRC. Thus, we investigated whether *PXDN* methylation status had an impact on CRC. We did not identify any correlation of *PXDN* methylation status in tumor tissue or adjacent normal tissue with CRC prognosis.

Our study has several advantages. The candidate gene approach was used in a rigorous approach comprising three steps. We further conducted quantitative analysis determined by what we observed from an MS-PCR. EpiTYPER DNA methylation analysis is a quantitative method of assessing methylation status. It also can confirm the degree of methylation at different CpG sites, which might be conductive to accurately identifying sites that affect CRC prognosis. This study was not without limitations. The results should be interpreted with caution on account of the small sample size. In the future, a larger prospective cohort study should be conducted to confirm our results. Furthermore, the participants were recruited from a single medical center in Taiwan. The effectiveness of the candidate genes as prognostic biomarkers for CRC should be validated in other ethnic populations. Finally, we did not detect the expression levels of selected genes, meaning we could not prove that the status or extent of methylation directly influenced gene expression.

CONCLUSION

Current clinical staging systems do not allow physicians to precisely evaluate the prognosis and outcomes of patients with CRC. Novel methylation biomarkers in tumor tissue and adjacent normal samples were identified in our study, providing a novel insight into how these markers could be used for CRC prognosis estimations. We anticipate that large-scale and independent cohort studies will clarify the utility of these novel markers and address whether clinical management can be adjusted based on supplementary information on the RFS, PFS, and OS of patients with CRC. We propose investigating these new biomarkers in patients with CRC to assist with clinical decision-making.

ARTICLE HIGHLIGHTS

Research background

The tumor–node–metastasis (TNM) cancer staging system provides clinical guidelines for the classification of tumors and prediction of outcomes. However, patients within the same stage can have markedly different outcomes. For example, some patients with an early disease stage of colorectal cancer (CRC) experience relapse after surgical treatment. Prognostic factors related to relapse or progression should be considered to improve treatment selection. The combination of several novel prognostic biomarkers involving epigenetic changes may aid CRC prognosis predictions.

Research motivation

To investigate the impact of the differential DNA methylation of novel candidate genes on CRC prognosis.

Research objectives

This study focused on the association between CRC prognosis and the status and level of differential DNA methylation of candidate genes.

Research methods

In total, 208 patients with CRC were recruited to assess the relationship between the methylation status of selected genes and clinical outcomes after surgical resection. The methylation statuses of *SUMF2*, *ADAMTS5*, and *PXDN* in tumor tissue and tumor-free adjacent areas were evaluated through a methylation-specific polymerase chain reaction (MS-PCR), and the methylation degrees of *SUMF2* and *ADAMTS5* were assessed using EpiTYPER DNA methylation analysis. The relationships of gene methylation with recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were evaluated using a Cox proportional hazards model and Kaplan–Meier survival curves.

Research results

CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue was associated with significantly shorter PFS and OS compared with CpG_3+CpG_7 hypomethylation. CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue was associated with a significantly longer RFS compared with CpG_2 and CpG_13 hypomethylation. No significant difference was noted in the association between the methylation status of *PXDN* in both tissue types and CRC prognosis.

Research conclusions

These results can be applied to develop useful prognostic biomarkers of CRC, especially the methylation of certain CpG islands of candidate genes. The results can add value to current cancer staging systems.

Research perspectives

Examining the differential DNA methylation of candidate genes could aid in clinical decision-making related to CRC. Further validation and investigations involving larger cohorts are required to confirm the utility of these new epigenetic biomarkers and determine whether they can be used to improve the RFS, PFS, and OS of patients with CRC.

FOOTNOTES

Author contributions: Hsu CH, Lin HC and Chou YC contributed equally to this work; Su JQ, Hsu CH, Lin HC and Chou YC designed the research; Sun CA and Yang T performed the research; Hu JM, Chang PK and Chen CY collected the data; Su JQ, Lai PY, Hu PH, Wu JJ and Lin YJ analyzed the data; Su JQ wrote the original draft; Hsu CH, Lin HC and Chou YC wrote the review and editing; Chou YC was the project administration.

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Informed consent statement: Written informed consent was obtained from all patients before enrollment into the study to evaluate their prognosis.

Conflict-of-interest statement: We have no financial relationships to disclose.

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Country/Territory of origin: Taiwan

ORCID number: Jing-Quan Su 0000-0001-9610-5736; Pin-Yu Lai 0000-0002-1093-7329; Pei-Hsuan Hu 0000-0002-3862-9707; Je-Ming Hu 0000-0002-7377-0984; Pi-Kai Chang 0000-0002-8641-3230; Chao-Yang Chen 0000-0002-2246-7635; Jia-Jheng Wu 0000-0002-5537-1162; Yu-Jyun Lin 0000-0003-1559-2259; Chien-An Sun 0000-0001-9041-0537; Tsan Yang 0000-0002-8265-6438; Chih-Hsiung Hsu 0000-0003-4423-4231; Hua-Ching Lin 0000-0003-3958-3760; Yu-Ching Chou 0000-0003-4823-6541.

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ORIGINAL ARTICLE

Retrospective Study

Long-term outcomes of endoscopic submucosal dissection and surgery for undifferentiated intramucosal gastric cancer regardless of size

Gil Ho Lee, Eunyoung Lee, Bumhee Park, Jin Roh, Sun Gyo Lim, Sung Jae Shin, Kee Myung Lee, Choong-Kyun Noh

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Gil Ho Lee, Sun Gyo Lim, Sung Jae Shin, Kee Myung Lee, Choong-Kyun Noh, Department of Gastroenterology, Ajou University School of Medicine, Suwon 16499, South Korea

Eunyoung Lee, Bumhee Park, Department of Biomedical Informatics, Ajou University School of Medicine, Suwon 16499, South Korea

Jin Roh, Department of Pathology, Ajou University School of Medicine, Suwon 16499, South Korea

Corresponding author: Choong-Kyun Noh, MD, PhD, Assistant Professor, Department of Gastroenterology, Ajou University School of Medicine, 164, Worldcup-ro, Yeongtong-gu, Suwon 16499, South Korea. cknoh23@gmail.com

Abstract

BACKGROUND

The clinical outcomes of endoscopic submucosal dissection (ESD) for undifferentiated (UD) intramucosal early gastric cancer (EGC) compared with those of surgery, regardless of lesion size, are not well known. Furthermore, there is a concern regarding the treatment plan before and after ESD in cases of UD intramucosal EGC within expanded indications.

AIM

To evaluate clinical outcomes of ESD compared with those of surgery in UD intramucosal EGC patients regardless of tumor size.

METHODS

We enrolled patients with UD intramucosal EGC after ESD with complete resection or surgery from January 2005 to August 2020 who met the within or beyond expanded indications with lesion size > 2 cm (the only non-curative factor). Overall, 123 and 562 patients underwent ESD and surgery, respectively. After propensity-score matching, clinical and long-term outcomes, *i.e.*, recurrence-free survival (RFS) and overall survival (OS), were analyzed. The multivariable Cox proportional hazard model with treatment modality and ESD indication was used to evaluate the recurrence risk.

RESULTS

After matching, 119 patients each were finally enrolled in the ESD and surgery groups. The median length of hospital stay was shorter in the ESD group than surgery group (4.0 *vs* 9.0 days, $P < 0.001$). Four cases of recurrence after ESD were local recurrences, all of which occurred within 1 year. Total recurrence was seven (5.9%) and two (1.7%) in the ESD and surgery groups, respectively. No difference was observed between the two groups with respect to OS ($P = 0.948$). However, the ESD group had inferior RFS compared with the surgery group ($P = 0.031$). ESD was associated with the risk of recurrence after initial treatment in all enrolled patients (hazard ratio, 5.2; 95% confidence interval: 1.0-25.8, $P = 0.045$).

CONCLUSION

Although OS was similar between the two groups, surveillance endoscopy was important for the ESD than for the surgery group because RFS was inferior and local recurrence was an issue.

Key Words: Early gastric cancer; Undifferentiated cancer; Expanded indication; Endoscopic submucosal dissection; Surgery

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Core Tip: This retrospective study evaluated the clinical outcomes of endoscopic submucosal dissection (ESD) compared with those of surgery in patients with undifferentiated (UD) intramucosal early gastric cancer (EGC) after propensity-score matching. No difference in overall survival was observed between two groups, although recurrence-free survival was inferior in the ESD group. Lymph node metastasis was not observed after ESD; however, local recurrence was higher after ESD than surgery. Surveillance endoscopy is important in ESD, even if complete resection is performed for UD intramucosal EGC. A short interval endoscopic follow-up is necessary when observing lesion sizes > 2 cm as the only non-curative factor.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is recommended as a treatment modality for early gastric cancer (EGC) because it allows curative *en bloc* resection and complete histopathological evaluation[1,2]. With the development of endoscopic instruments and techniques, the indication for ESD has expanded, and short- and long-term outcomes of ESD have been favorably reported in various studies[3-8]. Accordingly, ESD can be performed for patients with undifferentiated (UD) intramucosal EGC without lymphovascular invasion when the lesion size is ≤ 2 cm and there is no ulceration. Compared with surgery, ESD may be an alternative treatment option for UD intramucosal EGC within expanded indications[9-12]; however, concerns regarding lymph node (LN) metastasis in patients with UD intramucosal EGC remain[12].

Even if UD intramucosal EGC meets the criteria of expanded indications, additional surgical treatment is recommended if the lesion size alone is a non-curative factor (lesion diameter > 2 cm)[2]. In this case, physicians are concerned about determining the appropriate treatment modality and whether additional treatment should be performed. Various risk factors for LN metastasis have been reported in several surgical reports based on a lesion size of 2 cm[13-16]. Therefore, the role of ESD is limited in patients with UD intramucosal EGC because of the lesion size. However, patients may choose ESD for several reasons such as refusal of surgical treatment or older age. A recent multicenter study reported that mortality was not significantly higher in patients who underwent endoscopic resection for UD intramucosal EGC with tumor size > 2 cm as the only non-curative factor than in those who underwent additional surgery[17].

Compared with surgery, ESD can reduce the period of hospital stay after treatment and improve quality of life. To date, no study has compared long-term outcomes between ESD and surgical treatment based on propensity-score matching in patients with UD intramucosal EGC who are within or beyond expanded indications but satisfy the criteria of curative resection except for lesion size. Thus, this study

aimed to evaluate the clinical outcomes and adverse events of ESD compared with those of surgery in patients with UD intramucosal EGC using propensity-score matching analysis. Furthermore, we compared long-term clinical outcomes of ESD and surgery after matching for patients with UD intramucosal EGC who are beyond the expanded indication but meet the criteria of curative resection, except for lesion sizes > 2 cm.

MATERIALS AND METHODS

Patients

We retrospectively analyzed patients who underwent ESD (n = 212) or surgery (n = 1373) for UD intramucosal EGC at the Ajou University Medical Center (Suwon, Republic of Korea) between January 1, 2005 and August 31, 2020. Among those patients, patients with included expanded indications and curative resection[2] were enrolled. Patients who satisfied the condition of curative resection but had a lesion size > 2 cm (beyond expanded indications, tumor size > 2 cm as the only non-curative factor) were also included. The expanded indications with curative resection for UD intramucosal EGC were described as follows: intramucosal tumor; UD type; without ulceration; *en bloc* resection; tumor-free lateral and deep resection margin; without lymphovascular (or LN) invasion; and lesion size ≤ 2 cm[2]. Exclusion criteria were as follows: previous history of gastric cancer; previous history of other malignancy; or initial multiple gastric cancers. Additionally, we excluded patients who underwent additional surgery after ESD. The study protocol was approved by Ajou University Hospital Institutional Review Board and Ethics Committee (Approval No. AJIRB-MED-MDB-21-101). The requirement for informed patient consent was waived owing to the retrospective nature of the study. All co-authors had access to study data and reviewed and approved the final manuscript.

ESD procedure and surgery

All ESD procedures were performed by expert endoscopists using single-channel (GIF-Q260J; Olympus, Tokyo, Japan) or two-channel (GIF-2TQ260M; Olympus) endoscopy. After identifying the lesion, circumferential marking was done 5 mm outside the tumor margin using a needle knife (Dual knife; Olympus) or argon plasma coagulation (Erbe Elektromedizin, Tübingen, Germany). Epinephrine mixed fluid (0.01 mg/mL) was injected into the submucosal layer to lift the lesion from the muscle layer, and dissection was performed using an insulated-tip knife (IT knife; Olympus). The resected specimen was retrieved using a Swirl Net (Olympus), and all samples were fixed in 10% buffered formalin solution and embedded in paraffin.

Patients underwent total or subtotal gastrectomy with LN dissection according to the treatment guidelines of the Japanese Gastric Cancer Association (2). Therefore, patients who were enrolled in the surgery group underwent laparoscopy-assisted or open gastrectomy with D1 or D1+β LN dissection. The surgeons decided on the extent of gastric resection according to the tumor location.

Gross and histopathologic evaluations

Tumor locations were categorized into upper, middle, or lower third of the stomach based on the longitudinal axis of the stomach. Endoscopic findings were classified into elevated, flat, and depressed according to the predominant type based on the Japanese Research Society for Gastric Cancer classification system[18]. A standard histopathological examination, including hematoxylin and eosin staining, was conducted. Tumor size, presence of ulceration, histologic type, depth of invasion, lymphatic and vascular invasions, and presence of tumor cells in the resection margin were assessed. Pathological diagnoses were made according to the Japanese Classification of Gastric Cancer[1].

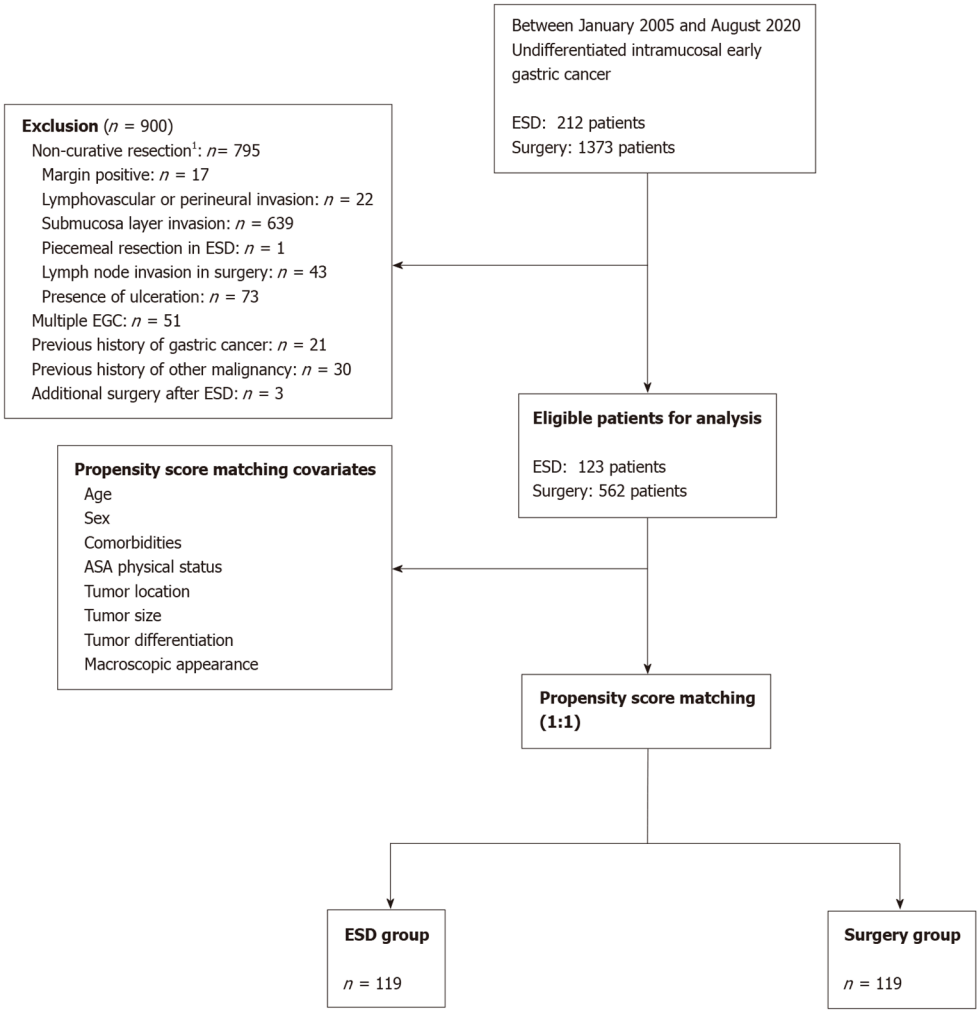
Follow-up schedules after gastric cancer resection

Follow-up endoscopy was performed 3 mo after ESD. A subsequent endoscopy with abdominal computed tomography (CT) was performed every 6-12 mo for 2 years and annually thereafter for 5 years after the treatment. In surgically resected patients, follow-up endoscopy and abdominal CT scans were performed every 6 mo for the first 2-3 years and then annually until 5 years after the initial treatment.

Clinical outcomes

The primary outcome of this study was overall survival (OS). The secondary outcomes were recurrence-free survival (RFS) and adverse events of short-term clinical outcomes. In this study, we defined OS as the duration between treatment and death owing to any cause; RFS was defined as the duration between treatment and first recurrence or death with evidence of recurrence. We collected data regarding survival status from the National Cancer Center (Goyang, South Korea); however, cause of death was not obtained for privacy after follow-up loss.

We defined local recurrence as a recurrence at the resection site after ESD or a recurrence at the anastomosis site after surgery. A synchronous lesion was defined as the occurrence of a new lesion



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Figure 1 Flow diagram of study population. [1]If the patient met within expanded indications except for lesion size factor > 2 cm (the only non-curative factor), we enrolled that patient for analysis. ASA: American Society of Anesthesiologists; EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection.

detected at a different site from the previous treatment site within 1 year after gastric cancer resection. A metachronous lesion was defined as the occurrence of a new lesion detected at a different site from the previous treatment site more than 1 year after initial treatment. Distant metastasis was defined as a tumor metastasis in another organ.

Statistical analysis

We performed propensity-score matching analysis using the radius method to balance covariates across groups and reduce selection bias in the observational study. The propensity score was estimated using a logistic regression model with seven matching variables such as age, sex, comorbidities, lesion size, tumor location, gross morphology, histology appearance, and American Society of Anesthesiologists (ASA) physical status classification system score. Based on these propensity scores, the ESD and surgery groups were matched in a 1:1 ratio on an allowable absolute difference between exact propensity scores. The standardized mean differences were computed to measure the balance of covariates between groups before and after propensity-score matching.

We compared demographics and clinical characteristics, clinical outcomes, and adverse events between the ESD and surgery groups using the independent *t*-test or Wilcoxon rank-sum test for continuous variables and Pearson's chi-square test or Fisher's exact test for categorical variables, as appropriate. Survival curves were plotted, and 5-year survival rates with 95% confidence intervals (CIs) were estimated using the Kaplan-Meier method. Differences in OS and RFS were examined using the log-rank test between the ESD and surgery groups and within and beyond the expanded indication and between the ESD and surgery groups among patients beyond the expanded indication separately. The multivariable Cox proportional hazard model with treatment modality and ESD indication was used to estimate the hazard ratio (HR) with 95%CIs to assess the recurrence risk.

All statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, United States), and R software, version 3.6.2 (R Project for Statistical Computing), and all P values < 0.05 were two sided and considered statistically significant.

RESULTS

Study population

In our center, 212 and 1373 patients with UD intramucosal EGC underwent cancer resection *via* ESD and surgery, respectively. Patients who failed to meet the expanded ESD indication and curative resection criteria, except lesion size, were excluded. For long-term outcome analysis, we finally enrolled 123 and 562 patients in the ESD and surgery groups, respectively. Before matching, the mean age ± SD of the ESD group was older than that of the surgery group (55.3 ± 12.4 *vs* 53.0 ± 11.8, P < 0.001). The proportions of male patients were 55.3% and 50.2% in the ESD and surgery groups respectively (P = 0.305). These two groups showed differences with respect to hypertension history, ASA physical status, tumor location, and histology type. In particular, the proportion of patients with signet ring cell carcinoma was higher in the surgery group than in the ESD group (73.1% *vs* 54.5%, P < 0.001); however, there was no significant difference between the two groups with respect to lesion size and ESD indication. There was also no significant difference between the ESD and surgery groups [43 (35.0%) *vs* 233 (41.5%), P = 0.183] with respect to the proportion of beyond expanded indications with lesion size > 2 cm (the only non-curative factor). We performed propensity-score matching to compare long-term outcomes of the ESD and surgery groups on a one-to-one basis, and all differences in baseline characteristics after matching were eliminated (Table 1). The flow diagram of enrolled patients is shown in Figure 1, and the distribution of propensity scores is shown in Supplementary Figure 1.

Clinical outcomes and adverse events

The median hospital stay [interquartile range (IQR)] was shorter in the ESD group than in the surgery group [4.0 (4.0-5.0) *vs* 9.0 (8.0-10.0) d, P < 0.001]. Regarding intensive care unit admission related to treatment complications, treatment complications caused by severe bleeding was noted one patient in the ESD group, who eventually died. Although the incidence of all adverse events was not different between the two groups, five cases (4.2%) of perforations and eight cases (6.7%) of bleeding occurred in the ESD group, all of which were early complications within 30 days. Surgical complications, including anastomotic leakage (n = 1, 0.8%), bowel obstruction (n = 3, 2.5%), and hernia (n = 3, 2.5%), were mostly successfully treated with conservative treatment; however, three hernia cases required additional surgery. Late complications were not observed in the ESD group; however, four cases (3.4%) were observed in the surgery group (Table 2).

Long-term outcomes

The median follow-up period was 45 mo (IQR, 21-65 mo) and 59 mo (IQR, 36-81 mo) in the ESD and surgery groups, respectively. During the follow-up period, three (2.5%) and five (4.2%) patients died in the ESD and surgery groups, respectively. Among those patients, gastric cancer-related death was identified in one patient (0.8%) in each group. The incidence of total recurrence was higher in the ESD group (n = 7, 5.3%) than in the surgery group (n = 2, 1.7%) (Table 3). Local recurrence was identified in four patients (3.4%) who underwent ESD, and all whom had EGC. A synchronous lesion was identified in one patient (0.8%), who had EGC and was treated with surgery. A metachronous lesion was identified in three patients (2.5%) of the ESD group. Distant metastasis was identified in one patient (0.8%) who underwent surgery with peritoneal metastasis. The number of patients whose tumor size was ≤ 2 cm before ESD or surgery and therefore, satisfied the criteria for expanded-indication lesions but had a size of > 2 cm in the final pathology analysis was 22 (18.5%) and 20 (16.8%) in the ESD and surgery groups, respectively. Of these patients, one patient in the ESD group had a recurrence but no mortality in both groups. Clinical and tumor data for all recurrent patients are given in Table 4.

We analyzed RFS and OS using Kaplan-Meier survival plots (Figure 2). Regarding RFS, according to the treatment modality, the ESD group had inferior results compared with the surgery group (P = 0.031) (Figure 2A). The 5-year RFS rates were 93.3% (95%CI: 85.1-97.0) and 99.2% (95%CI: 94.2-99.9) in the ESD and surgery groups, respectively. However, there was no significant difference between the two groups with respect to OS (ESD *vs* surgery, 5-year OS, 97.2%; 95%CI: 91.6-99.1 *vs* 99.0%; 95%CI: 93.0-99.9, P = 0.948) (Figure 2B). Among non-curative factors, we analyzed RFS (within *vs* expanded, 5-year RFS, 97.7%; 95%CI: 93.0-99.3 *vs* 94.6%; 95%CI: 85.6-98.0, P = 0.777) and OS (within *vs* expanded, 5-year OS, 98.5%; 95%CI: 94.1-99.6 *vs* 97.4%; 95%CI: 90.1-99.4, P = 0.698) for patients with lesion size > 2 cm (beyond the expanded indication but meeting the criteria of curative resection except for lesion size > 2 cm) and within expanded indication, in which no difference according to the indication was observed (Figure 2C and D). While there was no difference in OS (ESD *vs* surgery, 5-year OS, 97.5%; 95%CI: 83.5-99.6 *vs* 97.7%; 95%CI: 84.9-99.7, P = 0.610) according to the treatment modality in patients with beyond expanded indication with lesion size > 2 cm only, the ESD group had a significantly lower RFS than the surgery group (5-year RFS, 86.2%; 95%CI: 64.9-95.0 *vs* 100.0%; 95%CI: 100.0-100.0, P = 0.013) (Figure 2E

Table 1 Baseline characteristics of the study population

Variables	Before matching			After matching		
	ESD (*n* = 123)	Surgery (*n* = 562)	*P* value	ESD (*n* = 119)	Surgery (*n* = 119)	*P* value
Age, yr, mean SD	55.3 12.4	53.0 11.8	< 0.001	56.6 11.9	55.6 11.9	0.546
Male, *n* (%)	68 (55.3)	282 (50.2)	0.305	67 (56.3)	57 (47.9)	0.194
Comorbidity, *n* (%)						
Hypertension	44 (35.8)	143 (25.4)	0.020	41 (34.5)	40 (33.6)	0.891
Diabetes	21 (17.1)	65 (11.6)	0.095	19 (16.0)	15 (12.6)	0.459
Cerebrovascular disease	8 (6.5)	18 (3.2)	0.113	7 (5.9)	2 (1.7)	0.171
Respiratory disease	7 (5.7)	29 (5.2)	0.811	6 (5.0)	6 (5.0)	-
Liver disease	3 (2.4)	26 (4.6)	0.275	3 (2.5)	3 (2.5)	-
Renal disease	2 (1.6)	4 (0.7)	0.294	1 (0.8)	1 (0.8)	-
ASA physical status[1], *n* (%)			0.022			0.254
1	101 (82.1)	503 (89.5)		100 (84.0)	106 (89.1)	
2	22 (17.9)	59 (10.5)		19 (16.0)	13 (10.9)	
Tumor location, *n* (%)			< 0.001			0.822
Upper third	8 (6.5)	55 (9.8)		8 (6.7)	6 (5.0)	
Middle third	97 (78.9)	321 (57.1)		93 (78.2)	93 (78.2)	
Lower third	18 (14.6)	186 (33.1)		18 (15.1)	20 (16.8)	
Lesion size, mm, *n* (%)			0.430			0.418
10	30 (24.4)	105 (18.7)		28 (23.5)	19 (16.0)	
10-20	50 (40.7)	224 (39.9)		50 (42.0)	49 (41.2)	
20-30	24 (19.5)	133 (23.7)		22 (18.5)	28 (23.5)	
> 30	19 (15.4)	100 (17.8)		19 (16.0)	23 (19.3)	
Gross morphology type[1], *n* (%)			0.315			0.760
Elevated	33 (26.8)	127 (22.6)		29 (24.4)	27 (22.7)	
Flat or depressed	90 (73.2)	435 (77.4)		90 (75.6)	92 (77.3)	
Helicobacter pylori infection, *n* (%)	64 (51.2)	316 (56.2)	0.397	61 (51.3)	60 (50.4)	0.897
ESD indication, *n* (%)			0.183			0.183
Within expanded indication	80 (65.0)	329 (58.5)		78 (65.5)	68 (57.1)	
Beyond expanded indication	43 (35.0)	233 (41.5)		41 (34.5)	51 (42.9)	
Histology appearance, *n* (%)			< 0.001			0.794
Poorly differentiated carcinoma	56 (45.5)	151 (26.9)		52 (43.7)	54 (45.4)	
Signet ring cell carcinoma	67 (54.5)	411 (73.1)		67 (56.3)	65 (54.6)	

[1]Physical status classification of the American Society of Anesthesiologists.
ESD: Endoscopic submucosal dissection; SD: Standard deviation.

and F). In the multivariable analysis, ESD was a significant risk factor for recurrence after cancer resection in patients with UD intramucosal EGC (HR, 5.2; 95%CI: 1.0-25.8, *P* = 0.045). The lesion included in the beyond expanded indication was not associated with recurrence risk. Moreover, ESD as a treatment modality increased the HR for recurrence in the beyond expanded indication with lesion size > 2 cm compared with the within expanded indication; however, this result was not statistically significant (Table 5).

Table 2 Clinical outcomes and adverse events of early gastric cancer resection according to treatment modality

Variables	ESD (*n* =119)	Surgery (*n* = 119)	*P* value
Median hospital stay, d (IQR)	4.0 (4.0-5.0)	9.0 (8.0-9.0)	< 0.001
ICU admission, *n* (%)	1 (0.8)	0 (0.0)	-
30-d readmission, *n* (%)	3 (2.5)[1]	2 (1.7)[2]	-
Operation-related death, *n* (%)	1 (0.8)	0 (0.0)	-
Complication, *n* (%)	14 (11.8)	7 (5.9)	0.110
Bleeding (early/late)	8/0	0/0	
Perforation (early/late)	5/0	N/A	
Pneumonia (early/late)	1/0	0/0	
Anastomosis site leakage (early/late)	N/A	1/0	
Adhesion or bowel obstruction (early/late)	0/0	1/2	
Hernia (early/late)	N/A	1/2	

[1]All cases were bleeding.
[2]Vomiting owing to anastomosis site stricture, and pain owing to hernia.
Early and late complications occurred within or later than 30 days after operation or procedure, respectively. ESD: Endoscopic submucosal dissection; ICU: Intensive care unit; IQR: Interquartile range; N/A: Not applicable.

Table 3 Incidence and characteristics of recurrent tumors after initial treatment (endoscopic submucosal dissection or surgery)

Variables	ESD (*n* = 119)	Surgery (*n* = 119)	*P* value
Recurrence, *n* (%)	7 (5.9)	2 (1.7)	0.171
Local recurrence	4 (3.4)	N/A	
Adenoma	0 (0.0)	N/A	
Cancer	4 (3.4)	N/A	
Differentiated	3 (2.5)	N/A	
Undifferentiated	1 (0.8)	N/A	
Synchronous lesion	0 (0.0)	1 (0.8)	
Adenoma	0 (0.0)	0 (0.0)	
Cancer	0 (0.0)	1 (0.8)	
Differentiated	0 (0.0)	0 (0.0)	
Undifferentiated	0 (0.0)	1 (0.8)	
Metachronous lesion	3 (2.5)	0 (0.0)	
Adenoma	1 (0.8)	0 (0.0)	
Cancer	2 (1.7)	0 (0.0)	
Differentiated	1 (0.8)	0 (0.0)	
Undifferentiated	1 (0.8)	0 (0.0)	
Distant metastasis	0 (0.0)	1 (0.8)	

ESD: Endoscopic submucosal dissection; N/A: Not applicable.

DISCUSSION

We comparatively analyzed the long-term outcomes of patients who underwent ESD and surgery for UD intramucosal EGC with complete resection regardless of lesion size using propensity score-matched analysis. ESD was similar to surgery in terms of OS; however, RFS in the ESD group was lower than that in the surgery group. In both the groups, LN metastasis was absent during the follow-up period, and

Table 4 Clinical and tumor information for recurrent patients

Age	Sex	Location	Size (mm)	Morphology	Histology	Initial treatment	Recurrence type	Pathology of recurred lesion	Recurrence location	Recurrence time (mo)	Treatment for recurred lesion
61	F	Middle 1/3	6	Flat	SRC	ESD	Metachronous lesion	Undifferentiated cancer	Lower 1/3	70	ESD
62	M	Middle 1/3	22	Flat	SRC	ESD	Local recurrence	Undifferentiated cancer	Middle 1/3	6	Surgery
68	F	Middle 1/3	68	Flat	PDA	ESD	Metachronous lesion	Differentiated cancer	Upper 1/3	50	ESD
46	M	Middle 1/3	60	Flat	PDA	ESD	Local recurrence	Differentiated cancer	Middle 1/3	3	Surgery
50	F	Middle 1/3	25	Depressed	PDA	ESD	Local recurrence	Differentiated cancer	Middl1 1/3	12	ESD
62	M	Middle 1/3	40	Elevated	PDA	ESD	Local recurrence	Differentiated cancer	Middle 1/3	6	Surgery
56	F	Lower 1/3	8	Flat	PDA	ESD	Metachronous lesion	Adenoma	Middle 1/3	23	ESD
66	M	Lower 1/3	15	Flat	PDA	Surgery	Synchronous lesion	Undifferentiated cancer	Upper 1/3	5	Surgery
64	M	Middle 1/3	10	Elevated	SRC	Surgery	Distant metastasis	Undifferentiated cancer	Peritoneum	122	Conservative care

ESD: Endoscopic submucosal dissection; PDA: Poorly differentiated adenocarcinoma; SRC: Signet ring cell carcinoma.

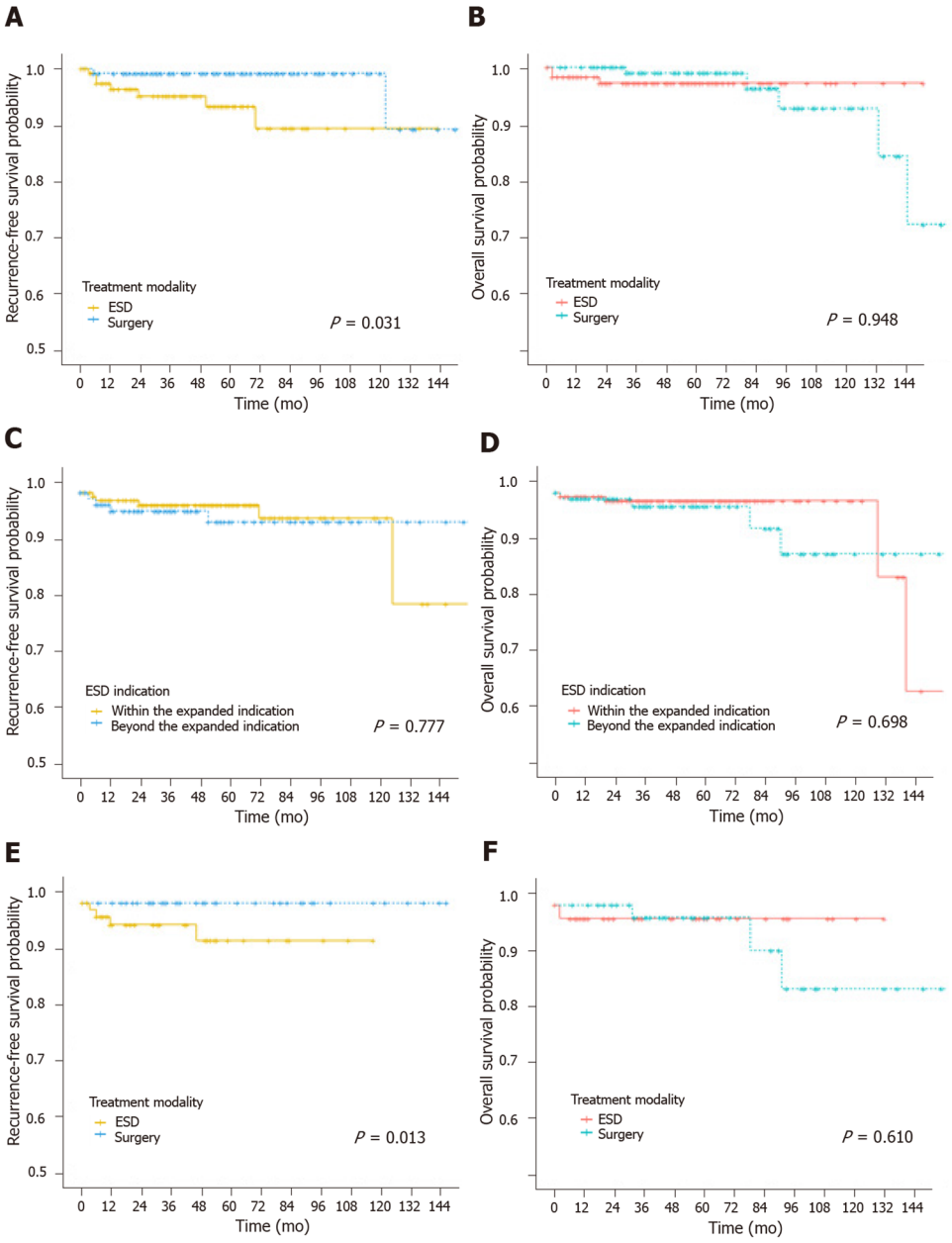
Table 5 Cox proportional hazard model for risk of recurrence after initial treatment

Variables	Adjusted hazard ratio (95% confidence interval)	*P* value
Treatment modality		
Surgery	1.0	
ESD	5.2 (1.0-25.8)	0.045
Indication with any treatment modality		
Within expanded indication	1.0	
Beyond expanded indication	1.4 (0.4-5.4)	0.585
Indication with ESD		
ESD for the lesion within expanded indication	1.0	
ESD for the lesion beyond expanded indication	2.8 (0.6-12.4)	0.183

ESD: Endoscopic submucosal dissection.

only one case of distant metastasis was observed in the surgery group. ESD was advantageous because of shorter hospital stays and fewer late complications compared with surgery. Although complete resection was performed for patients with UD intramucosal EGC, ESD was identified as a recurrence risk factor in terms of treatment modality compared with surgery. In patients with lesion size > 2 cm as the only non-curative factor, there was a difference in RFS depending on the treatment modality, with ESD having an inferior outcome.

In our study, there was a clear bias in the patient population depending on treatment modality. Before matching, patients in the ESD group were older and had a higher ASA physical status score, fewer lesions located in the upper third of the stomach, and lower signet ring cell carcinoma rate than those in the surgery group. While the expanded indication proposed by Gotoda *et al*[19] is based on the pathology of specimens, clinicians should select the treatment modality for EGC based on gross and pathological findings. However, this method sometimes makes it difficult to determine a treatment modality in clinical practice. Besides, there are cases in which patients beyond the expanded indication of ESD refuse surgery and thus undergo ESD in hopes of endoscopic resection instead of surgery. In



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Figure 2 Kaplan-Meier survival plots for recurrence-free survival and overall survival according to treatment modality for gastric cancer.
A: Recurrence-free survival (RFS), endoscopic submucosal dissection (ESD) *vs* surgery; B: Overall survival (OS), ESD *vs* surgery; C: RFS, within the expanded indication *vs* beyond expanded indication; D: OS, within the expanded indication *vs* beyond expanded indication; E: RFS, ESD *vs* surgery in patients with beyond expanded indication; F: OS, ESD *vs* surgery in patients with beyond expanded indication. ESD: Endoscopic submucosal dissection.

contrast, there are a significant number of patients who meet the expanded indications and undergo surgery. As a result, patients and lesion characteristics are not the same between patients who undergo ESD and those who undergo surgery. Our results suggested that patients were hesitant to undergo surgery if they were older or if their ASA physical status score was high. For this reason, selection bias was inevitable in this study group. Therefore, we performed propensity-score matching analysis to reduce selection bias owing to treatment modality.

With the development of ESD techniques and instruments and the accumulation of various clinical outcomes, the scope of ESD has been gradually expanding. However, issues regarding the role of ESD in patients with UD intramucosal EGC are continuously raised. Currently, the ESD criteria for UD intramucosal EGC are strict. ESD is performed only when the lesion has no ulceration and is ≤ 2 cm and when it satisfies the complete resection conditions of negative resection margins and the absence of lymphovascular and perineural invasion in the final pathological examination[2]. Lesion size > 2 cm is a non-curative factor, and various studies have reported a difference in LN metastasis based on lesion size of 2 cm[13,14,15,20]. However, in cases of UD-EGC with a lesion size > 2 cm, LN metastasis was reported to be 0% (0/54) when neither ulceration nor lymphovascular invasion was present[16], all of

which were from surgical studies. To our knowledge, only one study analyzed LN metastasis in patients who underwent endoscopic resection for UD intramucosal EGC with lesion size > 2 cm. Yang *et al*[17] reported an incidence of 1.1% (2/176), which showed no increase in mortality during observation after ESD based on Cox regression analysis. Similar results were also obtained in our study. LN metastasis was not observed among 119 patients who underwent ESD. However, 71.4% (5/7) of the total recurrence cases had a lesion size > 2 cm, which revealed a higher recurrence rate than that in patients with a lesion size ≤ 2 cm (11.9% *vs* 2.6%). Furthermore, patients with lesion size > 2 cm in the ESD group had a lower RFS than those in the surgery group.

The results of our analysis after matching for UD intramucosal EGC patients with complete resection, irrespective of lesion size, showed that RFS was inferior in the ESD group compared with the surgery group. However, no LN metastasis or distant metastasis was identified during the follow-up period. In other words, all recurrence cases occurred in the stomach, all were treated successfully after recurrence, and no further recurrences occurred during the follow-up period. However, taking ESD into account over surgery as the preferred treatment for UD intramucosal EGC irrespective of lesion size should be carefully considered. LN metastasis was not observed; however, local recurrence was found in four patients (4/119, 3.4%), all of whom had an initial tumor size > 2 cm that occurred less than a year after initial treatment, and surgery as a rescue treatment was performed because endoscopic treatment was impossible. In the study by Yang *et al*[17], the local recurrence rate was 2.3% (4/176), and one case had LN metastasis. Therefore, ESD must overcome the problem of local recurrence, which does not need to be considered in surgery. Furthermore, we should consider the why local recurrence occurs even after complete resection. The initial pathologic evaluation could have been incorrect or a new cancer may have occurred. We repeated the pathologic evaluation for these cases; however, the initial diagnosis did not change. Additional studies and data accumulation are required to examine why local recurrence occurs within a short period, although complete or curative resection is performed after the initial pathologic evaluation. In this study, in the 212 patients considered, the margin negative resection rate in the ESD group before matching was 92.0% (195/212). This was similar to previous studies where endoscopic resection was performed in UD EGC[21-26].

Therapeutic endoscopists always consider that a sufficient lateral margin can reduce the possibility of local recurrence. While it would be best to secure as much safety margin as possible, the operator should consider the duration of intervention, acute complications (bleeding or perforation), or delayed complications (bleeding, stricture). These considerations may be more prominent in UD EGC. A recently published study mentioned that local recurrence may be related to sequential molecular changes in various cancer-related proteins in histological margin-free endoscopically resected EGCs[27]. In this study, a tumor-free distance of 5.5 mm was considered insufficient as a safety margin. Besides, a subepithelial spread beneath the normal mucosa may exist in UD EGC, especially in signet ring cell cancer, and this subepithelial spread could reach up to 6 mm[28]. These studies suggest that securing sufficient margin in the endoscopic resection of UD EGC using the ESD method might reduce the rate of local recurrence. In the endoscopic resection of UD EGC using the ESD method, the endoscopically predicted and the actual size of the lesion is often different. In the Japanese algorithm, an additional biopsy was recommended from the surrounding mucosa of UD EGC to accurately evaluate the margin of the lesion[29]. In addition, other studies have reported that narrow-band imaging with magnifying endoscopy may help in accurately predicting the tumor extent in UD EGC[30]. Prospective randomized studies are required to evaluate whether the various attempts to accurately determine the tumor margin and resection with sufficient margin can reduce the rate of local recurrence.

In our study, all recurrence cases in the ESD group occurred in the lower or middle third of the stomach, except for one case (6/7, 85.7%). Although it is difficult to judge because of only a small number of recurrence cases, there is a report that incidence increased in the middle and lower third of the stomach in metachronous cancer after ESD[31]. This may be why fewer synchronous or metachronous recurrences were observed in the surgery group. In our study, 16.8% of patients underwent total gastrectomy after matching (20/119). Since the portion of the remnant stomach is small, even in patients who have undergone subtotal gastrectomy, surgery can be advantageous in terms of recurrence. In our study, only one recurrence occurred in the upper third of the stomach in the ESD and surgery groups. It is challenging to select ESD as the initial treatment option if surgical treatment is performed as a rescue treatment in a short period, given that ESD has a probability of local recurrence. Therefore, our results suggest that ESD should not be actively recommended to patients with UD intramucosal EGC with lesion size > 2 cm even without ulceration on preoperative workup.

This study had some limitations. First, this was a retrospective single-center study. A randomized study is required to compare long-term outcomes of ESD and surgery, but this is difficult to perform for UD intramucosal EGC. Second, baseline characteristics and tumor information were different between the groups; however, we analyzed the data after propensity-score matching to minimize the difference between baseline characteristics and reduce selection bias of treatment modality. Third, the number of patients with UD intramucosal EGC with lesion size > 2 cm was significantly lower in the pre-matching ESD group than in the surgery group (43 *vs* 233 patients). Although the data were corrected as much as possible with propensity-score matching, most ESD patients were assigned after matching; thus, selection bias could exist. Finally, although we confirmed survival based on data from the National Cancer Center registry, we did not check the cause of the death in all patients who died. Therefore, we

did not evaluate gastric cancer-related deaths in both groups after follow-up loss.

CONCLUSION

In conclusion, ESD is a treatment modality for stomach preservation with fewer late complications and shorter hospital stays than surgery. For patients with UD intramucosal EGC, if the lesion size is the only non-curative factor, ESD may be an alternative treatment option when surgery is not possible. However, all cases of local recurrence were identified within 1 year in our study, all of which were cancer, although LN metastasis was not observed after ESD. Therefore, even for complete resection, endoscopic surveillance is essential. Especially in cases with lesion sizes > 2 cm, endoscopic surveillance should be more thoroughly performed.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) is performed as an alternative treatment modality for undifferentiated (UD) intramucosal early gastric cancer (EGC) who are within the expanded indication. However, the ESD role for UD intramucosal EGC with lesion size > 2 cm (the only non-curative factor) is still controversial compared with surgery.

Research motivation

Several studies showed ESD could be performed for patients with UD intramucosal EGC within the expanded indication. However, the role of ESD is limited in these patients because of the lesion size. Even if UD intramucosal EGC meets the criteria of expanded indications, additional surgical treatment is recommended if the lesion size alone is a non-curative factor (lesion diameter > 2 cm).

Research objectives

In this study, the authors compared ESD with surgery in patients with UD intramucosal EGC who meet both the within expanded indications or beyond expanded indications with lesion size > 2 cm (the only non-curative factor).

Research methods

The authors retrospectively analyzed patients with UD intramucosal EGC after ESD with complete resection or surgery. After propensity-score matching, clinical outcomes and long-term outcomes, *i.e.*, recurrence-free survival (RFS) and overall survival (OS), were analyzed.

Research results

After propensity-scored matching, although ESD with complete resection was performed in UD intramucosal EGC regardless of lesion size, RFS increased, while there was no difference in OS compared to surgery. Especially, all cases of local recurrence were identified within 1 year in our study in the ESD group.

Research conclusions

Although ESD may be an alternative treatment option when surgery is not possible for UD intramucosal EGC with lesion sizes > 2 cm, endoscopic surveillance should be carefully performed within one year for local recurrence.

Research perspectives

Multicenter randomized studies with large cohorts are expected to evaluate ESD in patients with UD intramucosal EGC regardless of tumor size.

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FOOTNOTES

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Institutional review board statement: The study protocol was approved by Ajou University Hospital Institution's Review Board and Ethics Committee (Approval No. AJIRB-MED-MDB-21-101).

Informed consent statement: Patients were not required to give informed consent because the analysis used anonymous clinical data obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: No potential conflicts of interest were disclosed.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Country/Territory of origin: South Korea

ORCID number: Gil Ho Lee 0000-0001-7695-0828; Eunyoung Lee 0000-0002-9440-9119; Bumhee Park 0000-0002-5271-1571; Jin Roh 0000-0002-3995-355X; Sun Gyo Lim 0000-0003-2045-5099; Sung Jae Shin 0000-0003-1849-4435; Kee Myung Lee 0000-0003-3785-693X; Choong-Kyun Noh 0000-0002-3607-8120.

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ORIGINAL ARTICLE

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Yoo Min Han, Su Jin Chung, Seokha Yoo, Jong In Yang, Ji Min Choi, Jooyoung Lee, Joo Sung Kim

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Yoo Min Han, Su Jin Chung, Jong In Yang, Ji Min Choi, Jooyoung Lee, Department of Internal Medicine and Healthcare Research Institute, Seoul National University Hospital Healthcare System Gangnam Center, Seoul 06236, South Korea

Seokha Yoo, Department of Anesthesiology and Pain Medicine, Seoul National University College of Medicine, Seoul 03080, South Korea

Joo Sung Kim, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul 03080, South Korea

Corresponding author: Su Jin Chung, MD, PhD, Professor, Department of Internal Medicine and Healthcare Research Institute, Seoul National University Hospital Healthcare System Gangnam Center, 39th floor, GFC building 152, Teheran Ro, Gangnam-gu, Seoul 06236, South Korea. medjsj7@snuh.org

Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) infection is known to prevent the occurrence of gastroesophageal reflux disease (GERD) by inducing gastric mucosal atrophy. However, little is known about the relationship between atrophic gastritis (AG) and GERD.

AIM

To confirm the inverse correlation between AG and the occurrence and severity of GERD.

METHODS

Individuals receiving health checkups who underwent upper gastrointestinal endoscopy at Seoul National University Healthcare System Gangnam Center were included. The grade of reflux esophagitis was evaluated according to the Los Angeles classification. Endoscopic AG (EAG) was categorized into six grades. Serologic AG (SAG) was defined as pepsinogen I ≤ 70 ng/mL and pepsinogen I/II ratio ≤ 3.0. The association between the extent of EAG and SAG and the occurrence and severity of GERD was evaluated using multivariate logistic regression analysis.

RESULTS

In total, 4684 individuals with GERD were compared with 21901 healthy controls.

In multivariate logistic regression analysis, advanced age, male sex, body mass index > 23 kg/m^2, presence of metabolic syndrome, current smoking, and alcohol consumption were associated with an increased risk of GERD. Seropositivity for *H. pylori* immunoglobulin G antibodies was associated with a decreased risk of GERD. There was an inverse correlation between the extent of EAG and occurrence of GERD: Odds ratio (OR), 1.01 [95% confidence interval (CI): 0.90-1.14] in C1, 0.87 (0.78-0.97) in C2, 0.71 (0.62-0.80) in C3, 0.52 (0.44-0.61) in O1, 0.37 (0.29-0.48) in O2, and 0.28 (0.18-0.43) in O3. Additionally, the extent of EAG showed an inverse correlation with the severity of GERD. The presence of SAG was correlated with a reduced risk of GERD (OR = 0.49, 95%CI: 0.28-0.87, P = 0.014).

CONCLUSION

The extent of EAG and SAG exhibited strong inverse relationships with the occurrence and severity of GERD. AG followed by *H. pylori* infection may be independently protect against GERD.

Key Words: Gastroesophageal reflux disease; Reflux esophagitis; *Helicobacter pylori*; Atrophic gastritis; Pepsinogen

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Core Tip: This is a retrospective study to evaluate the inverse correlation of atrophic gastritis and the occurrence and severity of gastroesophageal reflux disease (GERD). Old age, male sex, body mass index over 23 kg/m^2, metabolic syndrome, current smoking, and alcohol intake increased the risk of GERD. Seropositivity for *Helicobacter pylori* (*H. pylori*) immunoglobulin G antibody decreased the risk of GERD. There was an inverse correlation between extent of endoscopic atrophic gastritis (EAG) and occurrence of GERD. Additionally, extent of EAG showed inverse correlation with severity of GERD. Presence of serologic atrophic gastritis was correlated with reduced risk of GERD. Atrophic gastritis followed by *H. pylori* infection could be an inde-pendent protective factor against GERD.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a disorder with specific symptoms, such as acid regurgitation and heartburn, caused by the recurrent reflux of gastric contents into the esophagus due to transient relaxation or low pressure of the lower esophageal sphincter[1,2]. A condition with troublesome symptoms accompanied by esophageal structural changes, such as mucosal breaks, erosion, or ulcers (*i.e.*, reflux esophagitis) is defined as erosive reflux disease (ERD); however, only about one-third to one-half of patients with GERD exhibit positive findings on endoscopic examinations[1]. Meanwhile, the presentation of typical esophageal symptoms in the absence of endoscopic changes is classified as non-ERD (NERD).

GERD is a common disease with a prevalence ranging from 18.1% to 27.8% in North America, 8.8% to 25.9% in Europe, and 2.5% to 7.8% in East Asia[3,4]. According to a systematic review conducted in 2014, the prevalence of GERD is significantly increased when comparing studies conducted before and after 1995[3]. Several factors such as a prolonged life expectancy, westernized lifestyle, and increasing prevalence of obesity might influence the increase in GERD prevalence[5]. GERD and its complications, such as reflux esophagitis and Barrett's esophagus, cause a considerable socioeconomic burden in terms of hospital visits, incur significant medical costs for diagnosis and treatment, and induce several problems related to quality of life. A number of epidemiological studies have evaluated the risk factors for GERD. Male sex, caffeine intake, smoking, alcohol consumption, dietary factors (especially the consumption of large meals and a high-fat diet), a low education level, and obesity are known risk factors for GERD[2,6-8].

Interestingly, an inverse correlation between GERD and *Helicobacter pylori* (*H. pylori*) infection has been demonstrated in several studies. In a case-control study performed in Japan, most cases of reflux esophagitis occurred in the absence of *H. pylori* and atrophic gastritis or in milder cases of gastritis with *H. pylori* infection[9]. *H. pylori* infection has been shown to reduce the risk and severity of GERD in

several epidemiological studies and systematic reviews[10-12]. Furthermore, the grade of reflux esophagitis and GERD symptoms are aggravated after *H. pylori* eradication therapy[2,13]. These findings suggest that *H. pylori* infection might be a protective factor against GERD as a result of a decreased acid secretory capacity induced by gastric mucosal atrophy.

As gastric mucosal atrophy is considered a key mechanism by which *H. pylori* infection prevents the occurrence or aggravation of GERD, several studies have assessed the relationship between atrophic gastritis and GERD. According to a few studies grading the severity of atrophic gastritis using endoscopic biopsies according to a modified updated Sydney classification, *H. pylori* infection alone may not influence the occurrence of reflux esophagitis. Meanwhile, the involvement and degree of atrophy in the gastric corpus are independent protective factors against GERD[9,14]. However, the histological diagnosis of atrophic gastritis based on endoscopic forcep biopsy is not always feasible in daily clinical settings because of its invasive nature. Pepsinogen is a validated serologic marker reflecting the acid secretory ability of the gastric gland; thus, it may predict the presence or absence of gastric atrophy[19]. In general, pepsinogen I levels ≤ 70 ng/mL and pepsinogen I/II ratios ≤ 3.0 are considered positive results for gastric atrophy[15]. A prospective case-control study in Korea found that the pepsinogen I/II ratio was higher in patients with ERD than in those without ERD[16]. Furthermore, the prevalence of reflux esophagitis decreased as the stage of *H. pylori*-related chronic gastritis assessed based on serum *H. pylori* antibody and pepsinogen levels progressed[17]. These studies showed that atrophic gastritis is a major preventive factor for GERD; however, the diagnosis of atrophic gastritis based on histological or serologic methods is not intuitive. In addition, these methods have limitations in terms of medical expenses, which cannot be easily applied in clinical trials.

Endoscopically, gastric atrophy is defined as a visible submucosal pattern in a non-overdistended stomach[18,19]. Previous epidemiological studies have revealed that the prevalence of endoscopic atrophic gastritis (EAG) and incidence of GERD show an inverse correlation[20-23]. However, these studies had several limitations; the extent of EAG was simplified into two types (closed and open types), and the definition of GERD was based solely on the presence of symptoms. Furthermore, these studies did not evaluate various confounding variables, such as demographic, metabolic, and lifestyle factors, which might interfere with the prevalence and grade of GERD.

In this study, we aimed to evaluate whether there is a quantitative correlation between the extent of atrophic gastritis and severity of GERD using endoscopic grading and serologic markers considering a variety of confounding variables in a large Korean population.

MATERIALS AND METHODS

Study population

Individuals receiving health checkups who underwent upper gastrointestinal (GI) endoscopy at Seoul National University Healthcare System Gangnam Center between January 2015 and December 2016 were screened for inclusion in the present study. We excluded those who had prior history of esophageal or gastric cancer and who performed esophagectomy or gastrectomy, active or healing stage of benign gastric or duodenal ulcer, and recent proton pump inhibitor (PPI) medication within one month. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the International Review Board of Seoul National University Hospital (IRB No. H-1701-028-655).

Clinical and laboratory assessment

Demographic data such as age, sex, height, weight, and waist circumference were collected. Physical examinations were performed using a written, systematic protocol with standardized instruments by trained personnel: The waist circumference were measured at midpoint between the lower border of rib cage and iliac crest[24]. Body mass index (BMI) was calculated using height and weight according to the formula: BMI = weight (kg)/height^2 (m^2). According to the modified World Health Organi-zation criteria from the Asia-Pacific guideline, BMI was categorized as follows: Normal (< 23 kg/m^2), overweight (23-24.9 kg/m^2) and obese (≥ 25 kg/m^2)[25].

Structured self-administered questionnaires were used to collect information including alcohol consumption (≥ 140 g/wk), current smoking (at least one cigarette per day for the previous 12 mo), current use of PPI (history of taking PPI within the last month), medication of sedatives or hypnotics and prior eradication therapy for *H. pylori* infection. We also assessed the types of physical activity and the time spent for each exercise. The MET values were assigned to physical activity data: 3.3 for walking at a moderate pace, 4.0 for moderate intensity, and 8.0 for vigorous intensity. The MET-minutes per week were estimated by multiplying the reported time spent at each activity by the corresponding MET value. Insufficient physical activity was defined when the MET value was under 3500 per week**.** Pattern of daily diet was also evaluated for having large meal, high fat diet, high salt diet or high caffeine intake.

Blood samples were drawn for the measurement of serum glucose, triglyceride, high-density lipoprotein cholesterol (HDL-C), *H. pylori* immunoglobulin G (IgG) antibody and pepsinogen I and II, in the morning after participants had fasted for at least 8 h.

Blood pressure was measured using an automated blood pressure monitor (TM-2655P, A&D Company, Saitama, Japan) twice after at least 5 min of rest in a seated position, and the mean value was used. High blood pressure was defined as a systolic blood pressure ≥ 130 mmHg or a diastolic blood pressure ≥ 85mmHg or taking anti-hypertensive medication. Hyperglycemia was defined as a fasting glucose ≥ 100 mg/dL or taking glucose lowering agents. Metabolic syndrome was diagnosed according to the National Cholesterol Education Program Adult Treatment Panel III criteria[26], if there are three or more of the following characteristics: High blood pressure, hyperglycemia, abdominal obesity (waist circumference ≥ 90 cm in men and ≥ 80 cm in women), hypertriglyceridemia (serum triglyceride level ≥ 150 mg/dL), and low HDL-C (HDL-C level < 40 mg/dL in men and < 50 mg/dL in women).

Endoscopic assessment

Seventeen experienced board-certified gastroenterologists performed all endoscopic examinations using conventional white light videoscopes (GIFH260 or GIFH290; Olympus, Aizu, Japan/EG-450WR5). All the gastroenterologists had more than 5 years of endoscopy experience (mean 12.1 years, range 5-27 years) and performed at least 2000 cases esophagogastroduodenoscopies each year.

Definition of GERD

The reflux esophagitis was defined if mucosal breaks or mucosal change such as erythema and/or discoloration were present. The grade of reflux esophagitis was categorized from M to D according to the Los Angeles (LA) classification system with Japanese modification which is based on the length of the longest mucosal break, and confluence of erosions[27,28]: N, normal mucosa; M, minimal changes to mucosa, such as erythema and/or whitish turbidity; A, non-confluent mucosal breaks no longer than 5 mm in length; B, non-confluent mucosal breaks more than 5 mm in length; C, confluent mucosal breaks less than 75% circumferential; D, confluent mucosal breaks at least 75% circumferential. NERD was diagnosed when a subject had the symptom of acid regurgitation or heartburn at a frequency of at least once per week in the absence of reflux esophagitis.

Definition of EAG

Gastric atrophy was evaluated endoscopically according to the location of endoscopic atrophy border (EAB), which is characterized by differences in color, visible capillary network, and height of the gastric mucosa[18,29]: "Closed type" indicates that the EAB is on the lesser curvature of stomach. On the other hand, in "open type", that is parallel to the vertical axis of stomach and extends along the anterior and posterior walls. The extent of EAG was categorized into six grades (C1 to O3): C0, no visible atrophy; C1, closed atrophy confined to the antrum; C2, closed atrophy confined to the antrum and lesser curvature of the distal gastric body; C3, closed atrophy involving the antrum and lesser curvature of the proximal gastric body; O1, open atrophy with the EAB placing between the lesser curvature and the anterior wall; O2, open atrophy with the EAB placing in the middle of the anterior wall; O3, open atrophy widely spread with the EAB between the anterior wall and the greater curvature.

Definition of serologic atrophic gastritis and H. pylori infection

A pepsinogen test was conducted for serological diagnosis of atrophic gastritis. Serum levels of pepsinogen I and pepsinogen II were measured by Latex Turbid immunoassay kits (HiSens Pepsinogen kit, HBI, Korea). Presence of serologic atrophic gastritis (SAG) was defined when pepsinogen I ≤ 70 ng/mL and pepsinogen I/II ratio ≤ 3[15].

H. pylori infection was measured by serum IgG antibodies using chemiluminescent microparticle immunoassay kits (Siemens, Germany). The values higher than 1.10 IU/mL were considered as a positive test for *H. pylori* infection.

Statistical analysis

For demographic and clinical characteristics of the study population, results were expressed as mean ± SD, median with interquartile range, or as counts with percentage. Pearson's chi-square test was used to compare categorical variables and an independent *t*-test was performed for continuous variables.

The association between EAG and GERD was evaluated using univariate and multivariate logistic regression analyses. First, the extent of EAG was categorized into 7 groups (normal to O3) and multivariate logistic regression analysis was performed to evaluate the risk of GERD according to the extent of EAG. It could show whether the extent of EAG influence the occurrence of GERD. Second, the risk of each grade of GERD (normal to LA-C/D) was independently analyzed according to the extent of EAG (normal to O3). It could show the relationship between extent of EAG and severity of GERD.

Pepsinogen I, II, and I/II ratio were compared using Kruskal-Wallis test due to their non-normal distributions, and post-hoc analysis was done with Wilcoxon rank sum test. In post-hoc analysis, *P*-value less than 0.0033 was considered statistically significant according to Bonferroni correction. To figure out the impact of SAG on the risk of GERD, multivariate logistic regression analysis was done.

The data were analyzed using R software version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics of the study population

A total of 27764 individuals underwent health checkup including screening upper GI endoscopy at Seoul National University Healthcare System Gangnam Center between January 2015 and December 2016. Among them, a total of 1179 were excluded from the study; 127 with a history of gastrectomy, 831 with current use of PPI, 157 with active or healing stage of gastric ulcer, 56 with active or healing stage of duodenal ulcer and 8 with esophageal cancer. Finally, a total of 26585 subjects were enrolled in this study and were divided into two groups [4684 (17.6%) in GERD group and 21901 (82.4%) in healthy group] according to the presence or absence of reflux symptom and esophageal structural change. The severity of GERD was graded as follows: NERD (1149 patients, 24.5%), LA-M (1229 patients, 26.2%), LA-A (1804 patients, 38.5%), LA-B (484 patients, 10.3%), LA-C (17 patients, 0.4%) and LA-D (1 patient, < 0.1%) (Figure 1).

Demographic and clinical characteristics of the study population were compared between the GERD and healthy groups (Table 1). Higher proportion of male was observed in GERD group (72.1% *vs* 53.1%, $P < 0.001$). Patients with general obesity (BMI > 25 kg/m^2) were more prevalent in GERD group (37.3% *vs* 25.0%, $P < 0.001$). Also, abdominal obesity (waist circumference ≥ 90 cm in men and ≥ 80 cm in women) were more common in GERD group (45.0% *vs* 35.6%, $P < 0.001$). Patients with GERD showed higher prevalence for high blood pressure (43.3% *vs* 35.6, $P < 0.001$), hyperglycemia (46.3% *vs* 38.6%, $P < 0.001$), hypertriglyceridemia (27.0% *vs* 18.9%, $P < 0.001$) and metabolic syndrome (30.7% *vs* 22.5%, $P < 0.001$). Excessive alcohol consumption and current smoking were more frequently observed in GERD group (21.6% *vs* 15.7%, $P < 0.001$ and 22.5% *vs* 15.4%, $P < 0.001$, respectively). Several life-style factors such as insufficient physical activity (33.7% *vs* 32.0%, $P = 0.022$), having large meal (12.3% *vs* 10.3%, $P < 0.001$), high fat diet (34.8% *vs* 31.3%, $P < 0.001$), high salt diet (13.3% *vs* 11.0%, $P < 0.001$), and high caffeine intake (30.5% *vs* 28.3%, $P = 0.003$) were more frequently accompanied in patients with GERD. Meanwhile, *H. pylori* antibody sero-positivity was less frequent in GERD group (25.3% *vs* 43.3%, $P < 0.001$).

Univariate and multivariate analysis on the risk for occurrence of GERD

Several variables on the risk for GERD were analyzed by univariate and multivariate logistic regression analysis (Table 2). All the variables included in the univariate analysis were used in multivariate analysis. Advanced age and male sex were significant risk factors for GERD in multivariate analysis [Odds ratio (OR) = 1.10, 95% confidence interval (CI): 1.06-1.14, $P < 0.001$ and OR = 1.96, 95%CI: 1.79-2.15, $P < 0.001$, respectively]. As BMI increased, the risk of GERD also increased (OR = 1.27, 95%CI: 1.16-1.39, $P < 0.001$ for BMI 23-25; and OR = 1.51, 95%CI: 1.37-1.67, $P < 0.001$ for BMI > 25). Metabolic syndrome was a significant risk factor for GERD (OR = 1.12, 95%CI: 1.03-1.22, $P = 0.008$). Excessive alcohol intake and current smoking also significantly increased risk of GERD (OR = 1.10, 95%CI: 1.00-1.21, $P = 0.043$; and OR = 1.26, 95%CI: 1.14-1.39, $P < 0.001$, respectively). Among various life-style factors including exercise and dietary habits, there were no significant risk factors for GERD occurrence. On the other hand, sero-positivity for *H. pylori* IgG antibody lowered the risk for GERD by approximately half (OR = 0.49, 95%CI: 0.45-0.54, $P < 0.001$).

Interestingly, there was an inverse correlation between extent of EAG and occurrence of GERD: As the extent of EAG progressed from normal to O3, the prevalence of GERD decreased gradually. In the multivariate logistic regression analysis, EAG was a significant protective factor for GERD: OR, 1.01 (95%CI: 0.90-1.14) in C1, 0.87 (0.78-0.97) in C2, 0.71 (0.62-0.80) in C3, 0.52 (0.44-0.61) in O1, 0.37 (0.29-0.48) in O2, and 0.28 (0.18-0.43) in O3. (Figure 2).

Correlation of EAG and severity of GERD

The grade of GERD in relation to the extent of EAG was evaluated and showed in Figure 3. The prevalence of GERD was highest in C1 and showed decreased tendency as extent of EAG progressed; 19.7% in normal, 22.1% in C1, 17.9% in C2, 13.3% in C3, 10.8% in O1, 8.2% in O2 and 8.1% in O3 (Figure 3A). As the extent of EAG progressed, the proportion of NERD gradually increased and proportion of ERD progressively decreased (Figure 3B).

To evaluate the impact of extent of EAG on the severity of GERD, subgroup analysis was performed (Figure 4). Grossly, as extent of EAG advanced, the risk of GERD decreased. This decreased tendency of OR for GERD intensified as the severity of GERD progressed from NERD to LA-B. Because the number of patients with LA-C/D was too small, it was impossible to calculate OR for LA-C/D.

Correlation of SAG and GERD

Among 26585 individuals included in this study, 2857 individuals (10.7%) underwent blood

Table 1 Demographic and clinical characteristics of the study population

	GERD group (*n* = 4684)	Healthy group (*n* = 21901)	*P* value
Age	50.9 ± 11.5	50.7 ± 11.1	0.330
Sex			< 0.001
Female	1305 (27.9%)	10265 (46.9%)	
Male	3379 (72.1%)	11636 (53.1%)	
BMI			< 0.001
< 23	1657 (35.4%)	11079 (50.6%)	
23-25	1278 (27.3%)	5346 (24.4%)	
≥ 25	1748 (37.3%)	5476 (25.0%)	
Abdominal obesity	2109 (45.0%)	7802 (35.6%)	< 0.001
High B	2028 (43.3%)	7795 (35.6%)	< 0.001
Hyperglycemia	2168 (46.3%)	8451 (38.6%)	< 0.001
Hypertriglycemia	1267 (27.0%)	4136 (18.9%)	< 0.001
Low-HDL	853 (18.2%)	4214 (19.2%)	0.108
Metabolic syndrome	1437 (30.7%)	4920 (22.5%)	< 0.001
Medication of sedatives or hypnotics	149 (3.2%)	704 (3.2%)	0.942
Alcohol consumption	1010 (21.6%)	3449 (15.7%)	< 0.001
Smoking history			< 0.001
Never smoker	2188 (46.7%)	12759 (58.3%)	
Ex-smoker	1444 (30.8%)	5764 (26.3%)	
Current smoker	1052 (22.5%)	3378 (15.4%)	
Insufficient physical activity	1578 (33.7%)	6998 (32.0%)	0.022
Having large meal	577 (12.3%)	2250 (10.3%)	< 0.001
High fat diet	1631 (34.8%)	6847 (31.3%)	< 0.001
High salt diet	624 (13.3%)	2414 (11.0%)	< 0.001
High caffeine intake	1427 (30.5%)	6200 (28.3%)	0.003
Seropositivity for *H. Pylori* IgG Ab	1068 (25.3%)	8453 (43.4%)	< 0.001

Ab: Antibody; BMI: Body mass index; GERD: Gastroesophageal reflux disease; HDL: High-density lipoprotein cholesterol; *H. Pylori*: *Helicobacter pylori*; IgG: Immunoglobulin G.

examination for pepsinogen test. There were minor and no significant differences in clinical characteristics including the prevalence of GERD between individuals with and without blood examination for pepsinogen test. Among them, 703 patients who underwent *H. pylori* eradication therapy were excluded and finally 2154 patients were included in the analysis. 358 patients (16.6%) had GERD and the severity of GERD was graded as follows: 78 patients (21.8%) with NERD, 79 patients (22.1%) with LA-M, 154 patients (43.0%) with LA-A, 46 patients (12.8%) with LA-B, and 1 patient (0.3%) with LA-C. Pepsinogen I level showed no significant association with the severity of GERD (P = 0.802). On the other hand, pepsinogen II level was significantly different according to GERD severity (P < 0.001). Post-hoc analysis revealed that LA-A showed significant lower level of pepsinogen II compared with normal group (normal *vs* LA-A, P < 0.001). In addition, pepsinogen I/II ratio was significantly higher in GERD group compared with normal group (normal *vs* LA-M, P =0.002; normal *vs* LA-A, P < 0.001; and normal *vs* LA-B, P = 0.002). LA-A group also showed significant higher pepsinogen I/II ratio than NERD (NERD *vs* LA-A, P < 0.001) (Figure 5).

SAG group showed lower prevalence of GERD than normal group (Figure 6). Total 343 out of 1889 individuals without SAG (18.2%) had GERD, in other hands, 15 out of 265 individuals with SAG (5.7%) had GERD. In the multivariate logistic regression analysis, the risk of GERD was adjusted for age, sex, BMI, metabolic syndrome, medication of sedatives or hypnotics, alcohol intake, smoking history, physical activity, dietary factor, and *H. pylori* IgG. Presence of SAG was correlated with reduced risk of

Table 2 Univariate and multivariate analysis on the risk for gastroesophageal reflux disease

	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age (10 yr)	1.01	0.99-1.04	0.319	1.10	1.06-1.14	< 0.001
Male sex	2.28	2.13-2.45	< 0.001	1.96	1.79-2.15	< 0.001
BMI						
< 23	Reference			Reference		
23-25	1.60	1.48-1.73	< 0.001	1.27	1.16-1.39	< 0.001
> 25	2.13	1.98-2.30	< 0.001	1.51	1.37-1.67	< 0.001
Metabolic syndrome	1.53	1.42-1.64	< 0.001	1.12	1.03-1.22	0.008
Medication of sedatives or hypnotics	0.99	0.83-1.18	0.906	1.01	0.83-1.22	0.919
Alcohol intake	1.47	1.36-1.59	< 0.001	1.10	1.00-1.21	0.043
Smoking history						
Never smoker	Reference			Reference		
Ex-smoker	1.46	1.36-1.57	< 0.001	0.97	0.88-1.06	0.439
Current smoker	1.82	1.67-1.97	< 0.001	1.26	1.14-1.39	< 0.001
Low level of physical activity	1.08	1.01-1.16	0.021	1.06	0.98-1.14	0.132
Having large meal	1.23	1.11-1.35	< 0.001	1.06	0.95-1.18	0.304
High fat diet	1.17	1.10-1.26	< 0.001	1.00	0.93-1.08	0.992
High salt diet	1.24	1.13-1.36	< 0.001	1.07	0.96-1.19	0.195
High caffeine intake	1.11	1.04-1.19	0.003	0.97	0.90-1.05	0.464
Seropositivity for *H. Pylori* IgG Ab	0.44	0.41-0.47	< 0.001	0.49	0.45-0.54	< 0.001

Ab: Antibody; BMI: Body mass index; CI: Confidence interval; OR: Odds ratio; *H. Pylori*: *Helicobacter pylori*.

GERD (OR = 0.49, 95%CI: 0.28-0.87, P = 0.014) (Figure 7).

DISCUSSION

In this study, we aimed to evaluate the association between atrophic gastritis assessed using endoscopy and the occurrence and severity of GERD adjusting for many confounding variables in a large population of individuals receiving health checkups. As a result, the severity of GERD was inversely correlated with the extent of EAG. After adjusting for several known risk factors for GERD, EAG remained an independent protective factor for the occurrence of GERD. Furthermore, GERD also exhibited a negative association with SAG based on measurements of serum pepsinogen I and II levels. It has been postulated that a decreased acid secretory ability induced by gastric mucosal atrophy lowers the risk of GERD development and progression.

Several previous studies have evaluated the negative association between *H. pylori* infection and GERD[9-12]. Meanwhile, our study directly focused on atrophic gastritis as a protective factor for GERD, which is a more fundamental and systematic approach to show how *H. pylori* infection affects GERD. Furthermore, we performed stratified analysis based on the extent of EAG and endoscopic grade of GERD, while previous studies only provided limited information and simplified analyses based on the presence or absence of atrophic gastritis or GERD. We assessed atrophic gastritis using two independent methods: Endoscopy and serology. Both methods showed consistent protective effects of atrophic gastritis on the occurrence of GERD. We graded atrophic gastritis endoscopically, which is a non-invasive and relatively reliable method to evaluate the extent of gastric mucosal atrophy[29-33]. Meanwhile, histology-based assessment of gastric mucosal atrophy requires tissue sampling with biopsy forceps, which may induce complications such as bleeding, pain, and inflammation. Endoscopic diagnosis of atrophic gastritis is intuitive and applicable in daily clinical settings. We also assessed SAG by measuring serum pepsinogen I and II levels. We excluded patients who underwent *H. pylori* eradication therapy because pepsinogens normalize after successful *H. pylori* eradication. It would be incorrect to evaluate atrophic gastritis merely by pepsinogens in cases after *H. pylori* eradication

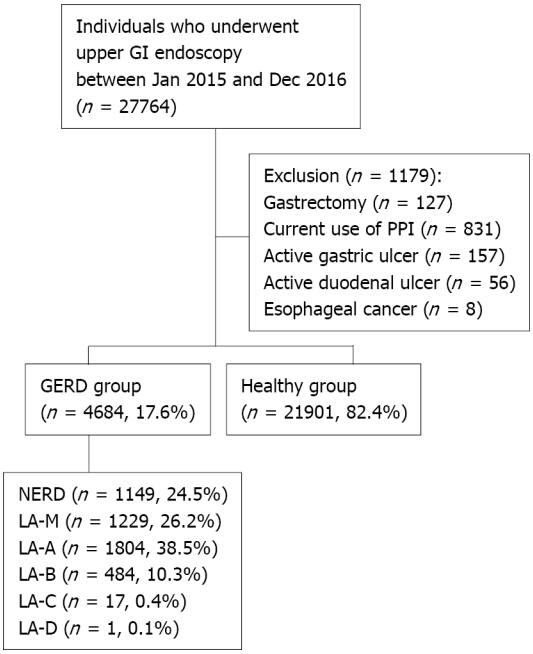


Figure 1 Flow diagram of the study enrollment. GERD: Gastroesphageal reflux disease; GI: Gastrointestinal; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; LA: Los Angeles.

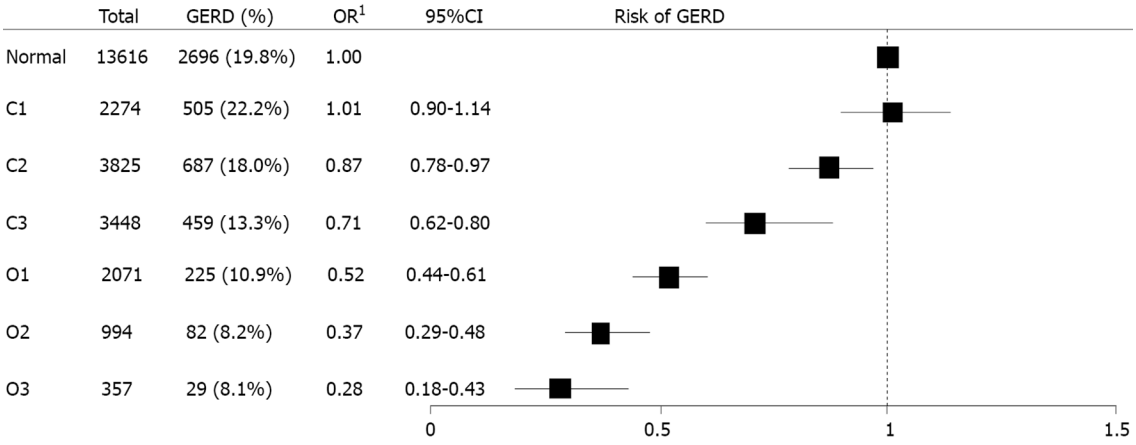


Figure 2 Multivariate analysis on the risk for occurrence of gastroesphageal reflux disease according to the extent of endoscopic atrophic gastritis. [1]Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G. CI: Confidence interval; GERD: Gastroesophageal reflux disease; OR: Odds ratio.

therapy. We found that the risk of GERD decreased as SAG progressed. This suggests that not only endoscopy but also simple blood tests may provide information about the risk and severity of GERD. However, interpretation of our findings requires careful consideration from several aspects. Since GERD can only be diagnosed endoscopically, picking up patients without SAG as GERD high risk would only increase the burden of excessive endoscopy and seems unnecessary. It may be sufficient to recommend endoscopy to patients with GERD-related symptoms. In order to prove the clinical usefulness of serological tests in GERD risk assistance, more research data is needed in future studies.

We investigated a variety of possible risk factors for GERD based on structured questionnaires. According to our study, several factors also influence the occurrence of GERD. Advanced age, the male sex, a high BMI, and the presence of metabolic syndrome were shown to be significant risk factors for GERD. Alcohol consumption and smoking were also associated with an increased GERD risk. A recent meta-analysis revealed that advanced age, a high BMI, a low education level, living in an urban area, current smoking, a low income, the consumption of carbonated drinks, and coffee/tea intake increase the risk of GERD, which is comparable with our results[4].

Interestingly, the seropositivity of *H. pylori* IgG was negatively correlated with GERD. Several previous studies have revealed that the prevalence of *H. pylori* infection is significantly lower in patients with GERD[10,11]. It is assumed that chronic inflammation induced by *H. pylori* results in gastric

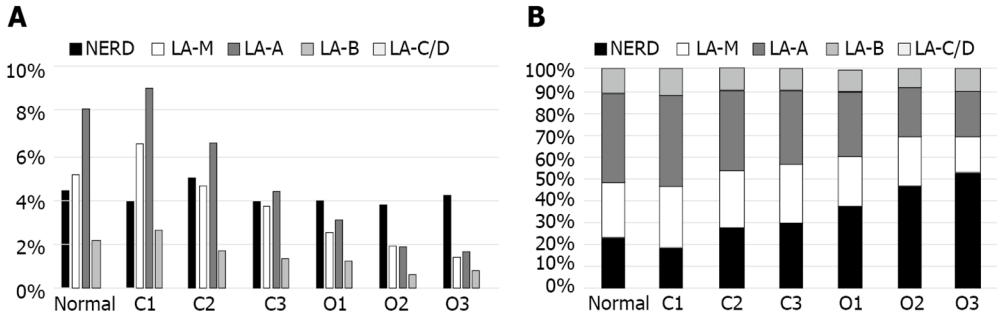


Figure 3 The grade of gastroesophageal reflux disease in relation to the extent of endoscopic atrophic gastritis. A: Prevalence of gastroesophageal reflux disease (GERD) according to the extent of endoscopic atrophic gastritis (EAG); B: Distribution of the severity of GERD according to the extent of EAG. NERD: Non-erosive reflux disease; LA: Los Angeles.

atrophy, which further decreases the acid secretory capacity of gastric mucosa. Therefore, the distribution and type of gastritis related to *H. pylori* are more important than the *H. pylori* infection itself[34]. Antrum-predominant gastritis induces hypergastrinemia and increased acidity; consequently, the risk of GERD increases in patients with antral gastritis[35]. In contrast, in cases of severe corpus gastritis, decreased gastric acid production is considered the main pathogenesis by which *H. pylori* infection protects against GERD[36]. Based on this, we evaluated the risk of GERD according to the extent of EAG. Interestingly, the risk of GERD was highest in association with C1 and gradually decreased as the extent of EAG progressed. This supports that atrophic gastritis rather than *H. pylori* infection itself is a key risk factor for GERD.

Our study has several advantages over other studies. First, we defined the extent and severity of atrophic gastritis using endoscopic and serologic methods, which enabled us to evaluate the effect of atrophic gastritis on the occurrence and severity of GERD. Second, we enrolled participants from a health screening cohort that represented the general population. For this reason, there was a minimal risk of selection or referral bias. The subjects were limited to individuals who had no specific disease and underwent regular endoscopic screening, as these conditions facilitated the assessment of the true impact of the disease. Third, high-quality data were obtained based on structured questionnaires and fully computerized electronic medical records, including data on demographics, laboratory examinations, family history, lifestyle factors, and most importantly, disease-specific symptoms. Healthwatch version 2.0, the large database in our center, fully computerizes a broad range of exposures including a comprehensive drug history and makes it possible to control for potential confounders, thereby permitting a less biased estimate of the association.

The interpretation of our findings requires careful consideration from several perspectives. First, the cross-sectional design prevents any conclusions regarding causality among *H. pylori* infection, atrophic gastritis, and GERD. Second, this study was conducted in subjects at a single healthcare center; therefore, the pool of subjects might represent a relatively high socioeconomic status, and it is possible that the enrolled individuals were more concerned about health. However, this study design is more favorable to other studies performed in tertiary hospitals, and our study population approached the general population. Third, *H. pylori* infection status was evaluated solely using a serologic test. There was no clinical information available regarding current or past *H. pylori* infection status in the serologic test. Nevertheless, serology is the most frequently used method for epidemiological research and has been used to predict the prevalence of *H. pylori* infection in various populations with approved sensitivity and specificity. Fourth, it would be better if we had performed 24-h esophageal pH monitoring tests for the diagnosis of GERD, which is considered the gold-standard method. Twenty-four-hour esophageal pH monitoring tests require specific medical devices, and it is an uncomfortable and time-consuming procedure for patients. At our institute, 24-h esophageal pH monitoring is not generally available in most clinics; thus, the diagnosis of GERD is based on subjective reports of symptoms and endoscopic examination results. Therefore, we believe our data reflect real-world practice. Finally, gastric atrophy was assessed using conventional white-light endoscopy. The diagnostic accuracy of conventional white-light endoscopy is reported to be relatively low compared with that of autofluorescence imaging[37,38].

The primary goal of *H. pylori* eradication therapy is to improve atrophic gastritis and to reduce carcinogenic risk and associated mortality, and it is clear from previous studies that eradication therapy can reduce cancer deaths[39,40]. Even if eradication therapy is likely to exacerbate the symptoms and clinical course of GERD, it seems clear that eradication treatment should be prioritized over GERD prevention. Another implication of our study is that clinicians should pay attention to asymptomatic patients with atrophic gastritis. Patients with GERD may experience diverse symptoms such as heartburn or acid reflux; therefore, they might have a high chance of visiting clinics to manage the symptoms and undergo thorough testing, such as upper GI endoscopy. However, patients with severe atrophic gastritis, even with a higher risk of gastric cancer[8,30], have a lower risk of developing GERD.

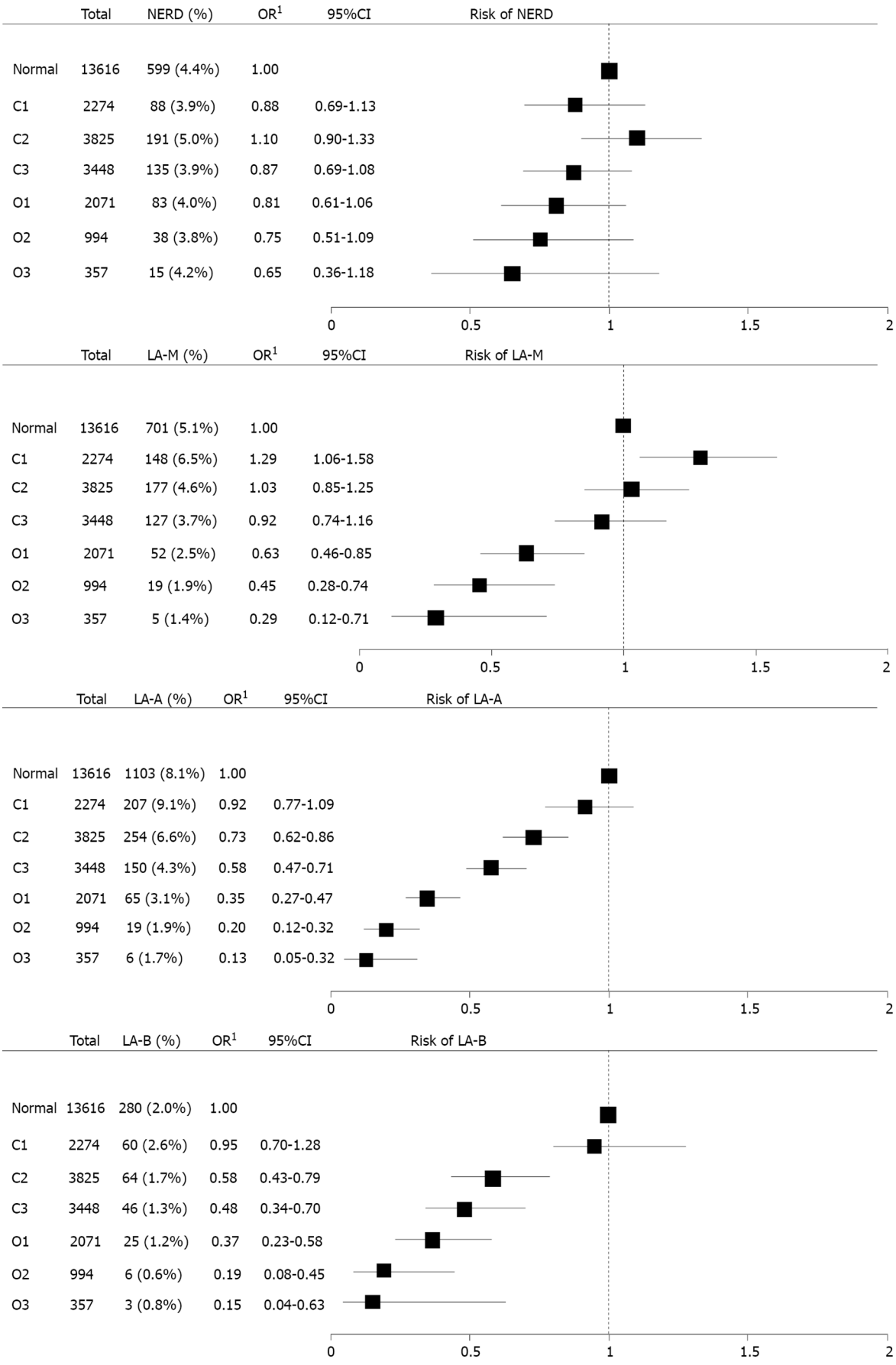


Figure 4 Impact of the extent of endoscopic atrophic gastritis on the risk for severity of gastroesophageal reflux disease. [1]Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G. NERD: Non-erosive reflux disease; CI: Confidence interval; OR: Odds ratio; LA: Los Angeles.

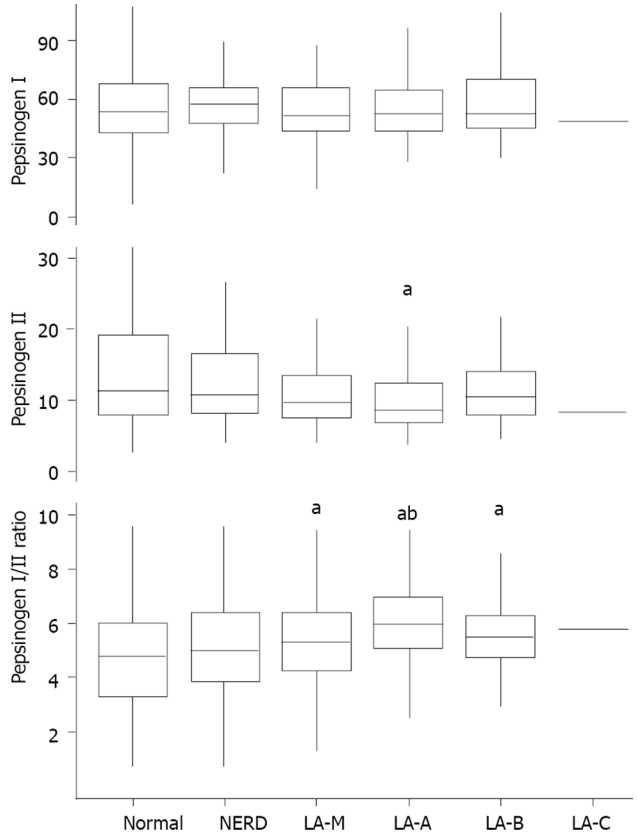


Figure 5 Pepsinogen I, II and I/II ratio according to the severity of gastroesophageal reflux disease. [a]*P* < 0.0033 compared to normal; [b]*P* < 0.001 compared to non-erosive reflux disease. NERD: Non-erosive reflux disease; LA: Los Angeles.

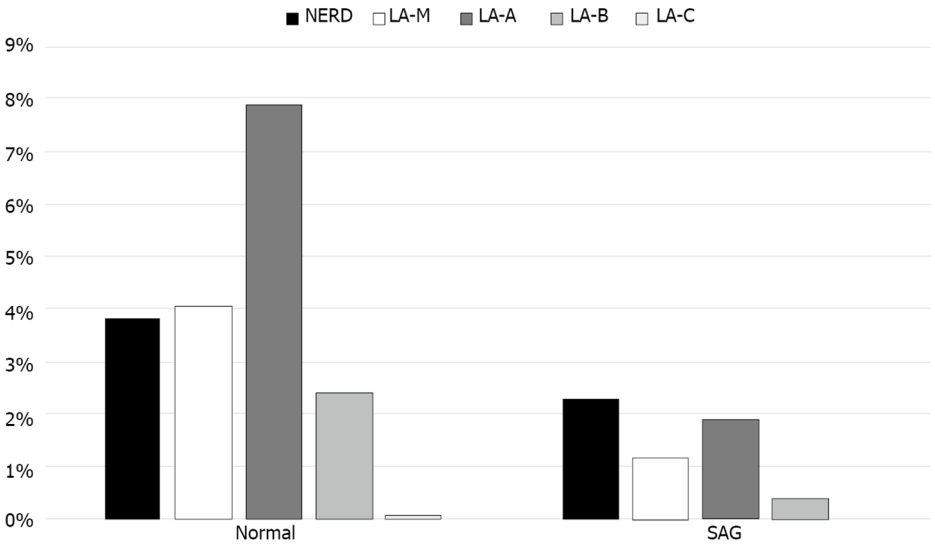


Figure 6 Prevalence of gastroesophageal reflux disease according to serologic atrophic gastritis. NERD: Non-erosive reflux disease; LA: Los Angeles; SAG: Serologic atrophic gastritis.

These individuals are less likely to visit a medical center on their own because they are free of GERD-related symptoms. Therefore, it is important to perform screening endoscopy in individuals without symptoms, especially in cases of extended EAG or SAG presented in previous examinations. Furthermore, if a patient with severe EAG complains of GERD-related symptoms such as heartburn or acid reflux, the possibility of other upper GI diseases including non-acid reflux or esophageal hypersensitivity should be considered because GERD is rarely accompanied by severe gastric atrophy. In the present patient, we considered a different approach than the routine prescrip-tion of PPIs.

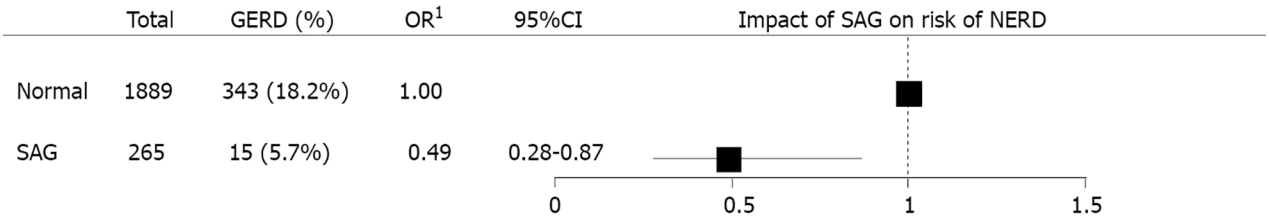


Figure 7 Multivariate analysis on the risk for occurrence of gastroesophgeal reflux disease according to serologic atrophic gastritis. [1] Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G Ab. NERD: Non-erosive reflux disease; CI: Confidence interval; SAG: Serologic atrophic gastritis; OR: Odds ratio.

Our findings must be confirmed through prospective clinical trials. The acid secretory activity of the gastric mucosa according to *H. pylori* status and extent of EAG should be further evaluated to determine the pathogenesis of GERD. In addition, an analysis of gastroesophageal motility based on *H. pylori* status and the extent of EAG would provide a better understanding of the mechanisms of GERD. Moreover, long-term interventional studies examining the effect of treating *H. pylori* infection on esophageal disease will provide clear information on which to base clinical decisions.

CONCLUSION

In summary, we found that the extent of EAG and SAG had strong inverse relationships with the occurrence and severity of GERD in a large sample of the Korean population after adjusting for multiple confounding factors. These data support the hypothesis that atrophic gastritis followed by *H. pylori* infection is an independent protective factor against GERD. As the number of patients with gastric atrophy may decrease based on the reduced *H. pylori* infection rate and broad application of *H. pylori* eradication therapy, the prevalence of GERD may increase. Appropriate diagnosis and treatment strategies are required based on the endoscopic findings and symptoms of patients.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) infection is known to prevent the occurrence of gastroesophageal reflux disease (GERD) by inducing gastric mucosal atrophy. However, little is known about the relationship between atrophic gastritis (AG) and GERD.

Research motivation

Our study directly focused on AG as a protective factor of GERD, which was more fundamental and systematic approach to show how *H. pylori* infection affects GERD.

Research objectives

We aimed to confirm the inverse correlation between AG and the occurrence and severity of GERD.

Research methods

We assessed AG in two independent methods, endoscopy and serology, furthermore, investigated variety of possible risk factors for GERD based on laboratory examination and structured questionnaires.

Research results

Advanced age, male sex, body mass index > 23 kg/m^2, presence of metabolic synd-rome, current smoking, and alcohol consumption were associated with an increased risk of GERD. Seropositivity for *H. pylori* immunoglobulin G antibodies was associated with a decreased risk of GERD. There was an inverse correlation between the extent of endoscopic AG (EAG) and occurrence of GERD. Additionally, the extent of EAG showed an inverse correlation with the severity of GERD. The presence of serologic AG (SAG) was correlated with a reduced risk of GERD.

Research conclusions

The extent of EAG and SAG exhibited strong inverse relationships with the occurrence and severity of GERD. AG followed by *H. pylori* infection may be independently protect against GERD.

Research perspectives

As the Kimura-Takemoto visual endoscopic method used in our study might be subjective, it would be better to continue further study using the endoscopic morphological method - Updated Kimura-Takemoto classification of AG. Furthermore, to clarify the causality between AG and GERD, prospective studies are warranted to follow how the prevalence and severity of GERD change according to the progression or regression of AG.

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FOOTNOTES

Author contributions: Han YM, Chung SJ designed and performed the research and wrote the paper; Yoo S designed the research and contributed to the analysis; Yang JI, Choi JM, Lee J collected the patients' clinical data and provided clinical advice; Kim JS provided clinical advice and supervised the report; and all authors have read and approve the final manuscript.

Institutional review board statement: This study was approved by the International Review Board of Seoul National University Hospital (IRB No. H-1701-028-655).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data. Written informed consent was waived by the Ethics Committee of the designated hospital.

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

Data sharing statement: Some or all data and code generated or used during the study are available from the corresponding author by request.

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Country/Territory of origin: South Korea

ORCID number: Yoo Min Han 0000-0001-5580-8427; Su Jin Chung 0000-0002-0983-8446; Seokha Yoo 0000-0003-4679-6027; Jong In Yang 0000-0002-1625-8271; Ji Min Choi 0000-0001-8611-4647; Jooyoung Lee 0000-0003-1652-088X; Joo Sung Kim 0000-0001-6835-4735.

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CASE REPORT

Treatment strategy for pancreatic head cancer with celiac axis stenosis in pancreaticoduodenectomy: A case report and review of literature

Eiji Yoshida, Yasutoshi Kimura, Takuro Kyuno, Ryoko Kawagishi, Kei Sato, Tsuyoshi Kono, Takehiro Chiba, Toshimoto Kimura, Hitoshi Yonezawa, Osamu Funato, Makoto Kobayashi, Kenji Murakami, Akinori Takagane, Ichiro Takemasa

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Eiji Yoshida, Takuro Kyuno, Ryoko Kawagishi, Kei Sato, Tsuyoshi Kono, Takehiro Chiba, Toshimoto Kimura, Hitoshi Yonezawa, Osamu Funato, Makoto Kobayashi, Akinori Takagane, Department of Surgery, Hakodate Goryoukaku Hospital, Hakodate City 040-8611, Japan

Yasutoshi Kimura, Ichiro Takemasa, Department of Surgery, Surgical Oncology and Science, Sapporo Medical University, Sapporo City 060-8543, Hokkaido, Japan

Kenji Murakami, Department of Diagnostic Radiology, Hakodate Goryoukaku Hospital, Hakodate City 040-8611, Japan

Corresponding author: Yasutoshi Kimura, MD, PhD, Associate Professor, Surgeon, Department of Surgery, Surgical Oncology and Science, Sapporo Medical University, S-1, W-16, Chuo-ku, Sapporo City 060-8543, Hokkaido, Japan. kimuray@sapmed.ac.jp

Abstract

BACKGROUND

During pancreaticoduodenectomy in patients with celiac axis (CA) stenosis due to compression by the median arcuate ligament (MAL), the MAL has to be divided to maintain hepatic blood flow in many cases. However, MAL division often fails, and success can only be determined intraoperatively. To overcome this problem, we performed endovascular CA stenting preoperatively, and thereafter safely performed pancreaticoduodenectomy. We present this case as a new preoperative treatment strategy that was successful.

CASE SUMMARY

A 77-year-old man with a diagnosis of pancreatic head cancer presented to our department for surgery. Preoperative assessment revealed CA stenosis caused by MAL. We performed endovascular stenting in the CA preoperatively because we knew that going into the operation without a strategy could lead to ischemic complications. Double-antiplatelet therapy (DAPT) – which is needed when a stent is inserted – was then administered in parallel with neoadjuvant chemotherapy (NAC). This allowed us to administer DAPT for a sufficient period before the main pancreaticoduodenectomy procedure while obtaining therapeutic effects from NAC. Subtotal stomach-preserving pancreaticoduodenectomy was then

performed. The operation did not require any unusual techniques and was performed safely. Postoperatively, the patient progressed well, without any ischemic complications. Histopathologically, curative resection was confirmed, and the patient had no recurrence or complications due to ischemia up to six months postoperatively.

CONCLUSION
Preoperative endovascular stenting, with NAC and DAPT, is effective and safe prior to pancreaticoduodenectomy in potentially resectable pancreatic cancer.

Key Words: Pancreaticoduodenectomy; Celiac axis stenosis; Median arcuate ligament; Endovascular stenting; Pancreatic head cancer; Case report

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Core Tip: Celiac axis stenosis (CAS), caused by the median arcuate ligament, is an anatomical anomaly that should be noted when performing pancreaticoduodenectomy. In this case, an endovascular stent was placed preoperatively to recanalize the stenotic celiac axis, allowing the patient to safely undergo radical surgery without concern for intraoperative organ perfusion. The point to be highlighted with this preoperative strategy, was the use of double-antiplatelet therapy following endovascular stenting, during the interval when for neoadjuvant chemotherapy for pancreatic cancer (PC), thus providing a rational and effective combination of the two preoperative treatments for patients with PC and CAS.

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INTRODUCTION

Celiac axis stenosis (CAS) – caused by the median arcuate ligament (MAL) – often hinders blood flow to the upper abdominal organs, particularly the liver, during pancreaticoduodenectomy[1-4]. This is because the gastroduodenal artery (GDA), which is responsible for maintaining hepatic blood flow during the procedure, needs to be dissected. The most common intervention to maintain hepatic arterial flow is intraoperative MAL division[1,5,6], which fails to recanalize the arterial flow in 30%-40% of cases [1,4,7]. Additionally, the success of MAL division can only be determined intraoperatively and, in case of failure, surgeons must choose to either undertake a complex revascularization or abandon the resection intraoperatively.

Recently, neoadjuvant chemotherapy (NAC) has become a common preoperative treatment for pancreatic cancer (PC)[8,9]. NAC improves the overall survival of patients with PC and is usually required for 2-3 mo, or more, depending on the necessary treatment regimen. To eliminate concerns about MAL compression prior to the scheduled pancreaticoduodenectomy, we undertook preoperative endovascular celiac axis (CA) stenting. Double-antiplatelet therapy (DAPT) was subsequently administered to avoid stent obstruction, with the NAC for PC continuing concurrently. Herein, we report on a reasonable and effective preoperative strategy that combines NAC for PC and endovascular stenting for MAL compression.

CASE PRESENTATION

Chief complaints

A 77-year-old man was referred to our department with a progressively enlarging pancreatic head mass.

History of present illness

The patient had been followed-up for intraductal papillary mucinous neoplasm of the pancreas for 20 years, with no significant changes in the periodically scheduled examinations until half a year ago.

In the last six months, a tumor of approximately 30 mm became visible on the pancreatic head and diagnosed as cancer after further investigation.

History of past illness

The patient had undergone robot-assisted laparoscopic prostatectomy for prostate cancer six years prior, as well as endoscopic submucosal dissection for early gastric cancer two years prior. He had a pacemaker implanted for atrioventricular block two years prior.

Personal and family history

The patient had no family history of PC or genetic disorders.

Physical examination

The patient's appetite was normal, and he had no weight loss or abdominal pain. Stool was normal with no history of constipation or diarrhea. Furthermore, jaundice was not observed.

Laboratory examinations

Laboratory results included an elevated carbohydrate antigen 19-9 (CA19-9) level of 308.5 U/mL, but the carcinoembryonic antigen level was normal. Other results, including diabetes indices such as HbA1c and glycoalbumin levels, were within normal limits. Bilirubin levels were also normal.

Imaging examinations

Computed tomography (CT) revealed a 30 mm pancreatic head tumor with poor enhancement in the early phase (Figure 1A). The tumor had not invaded the major vessels including the common hepatic artery, CA, and the superior mesenteric artery (SMA) and superior mesenteric vein. No metastatic lymph nodes and metastases to distant organs were apparent. Three-dimensional reconstruction images showed developed collateral pathways around the pancreatic head (Figure 1B and C). In the sagittal view, the CA was compressed by the MAL, which developed caudally (Figure 1D). Endoscopic retrograde cholangiopancreatography revealed stenosis in the main pancreatic duct (MPD) and biopsy from the stenotic site of the MPD detected adenocarcinoma.

FINAL DIAGNOSIS

He was diagnosed with cT2N0M0 (8th edition of the UICC-TNM classification) pancreatic head cancer comorbid with CAS caused by MAL.

TREATMENT

A pancreaticoduodenectomy was planned due to the diagnosis of pancreatic head cancer. To secure hepatic blood flow, endovascular stenting of the CA was undertaken prior to the surgery. After successful endovascular stenting, DAPT was administered to avoid stent obstruction, with NAC for PC continuing concurrently.

On aortography, the CA could not be visualized, and severe stenosis of the CA was suggested (Figure 2A). It was difficult to achieve an antegrade approach to the CA directly from the aorta because the root of the CA was severely stenotic, and the root was strongly bent. Therefore, we planned to approach the CA retrogradely from the SMA *via* the collateral pathway.

The procedure was initiated by placing sheaths in the right and left common femoral arteries. A 4Fr shepherd hook catheter (Angiomaster^{R}, Terumo, Tokyo, Japan) was selectively inserted into the SMA through the left sheath. Next, we used a triple-coaxial system to reach the CA[10]. In the triple coaxial system, a 2.7 Fr high flow catheter (Bishop HF, Piolax Medical Devices, Yokohama, Japan), a 1.9 Fr micro catheter (Carnelian^{R} Marvel^{R}, Tokai Medical Products, Aichi, Japan), and a 0.014-inch micro guidewire (Jupiter™ FC, Boston Scientific, Marlborough, MA, United States) were used. Because the guidewire was as fine as 0.014-inch, it was possible to break through the stenosis of the CA (Figure 2B). The microguidewire that reached the aorta was grasped with a snare (Atrieve™ Vascular Snare Kit, Argon Medical Devices, TX, United States) inserted through the right sheath and pulled out of the body. Then, a guiding sheath (Parent Plus^{R}60, Medikit, Tokyo, Japan) was inserted into the CA using the pull-through technique. A balloon expandable stent (Express™ LD vascular stent, Boston Scientific, Massachusetts, United States) was successfully deployed in the CA and an additional stent was used to reinforce the stenotic lesion. Finally, stent dilation was performed with a balloon (Mustang™ Balloon Dilatation Catheter, Boston Scientific, Massachusetts, United States). Aortography demonstrated CA patency and antegrade flow, and the procedure was completed (Figure 2C).

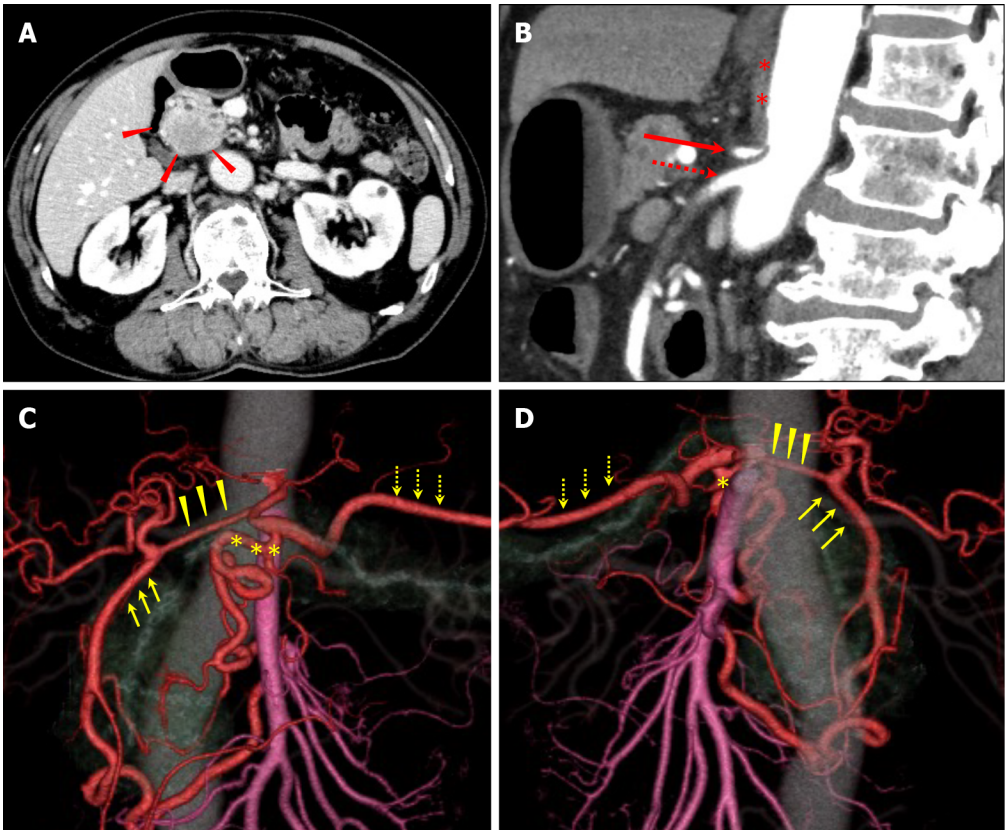


Figure 1 Pre-treatment imaging findings. A: There was a tumor, with poor contrast, in the pancreatic head on computed tomography (CT) imaging. Red arrowheads: the tumor; B and C: Three-dimensional reconstruction imaging showed developed collateral pathways around the pancreatic head. One connected the superior mesenteric artery (SMA) and common hepatic artery (CHA) *via* the gastroduodenal artery (GDA) and another connected the SMA and splenic artery (SPA) *via* the dorsal pancreatic artery (DPA); D: The sagittal view of the CT showed celiac axis (CA) stenosis due to compression by MAL which developed caudally. Yellow arrows: GDA; yellow arrowheads: CHA; yellow asterisks: DPA; yellow dotted arrows: SPA; red asterisks: MAL; red arrow: CA; red dotted arrow: SMA.

DAPT was started the day after stent insertion, and NAC was introduced on day 5. DAPT consisted of oral 100 mg aspirin and 75 mg $P2Y_{12}$ inhibitor once daily. NAC regimen was gemcitabine plus S-1 therapy, which consisted of intravenous gemcitabine at a dose of 1000 mg/m^2 on days 1 and 15 and S-1 orally at a dose of 60 mg twice daily on days 1-14 of a 28-d cycle. DAPT continued for 12 wk and two courses of NAC were administered. The preoperative course is shown in Figure 3.

After completing preoperative treatment, subtotal stomach-preserving pancreaticoduodenectomy was performed (Figure 4A). Since the CA flow was sufficient before the surgery, the surgical procedure was routinely performed, without any special intraoperative techniques. After clamping the GDA, we confirmed good blood flow in the proper hepatic artery (PHA) by palpation, then dissected the GDA. There was no need to preserve the collateral pathways that continued to the DPA or GDA, so we dissected them and obtained an adequate SMA-margin. When dissecting these collateral pathways, we confirmed that the PHA flow remained pulsatile as an indicator that the blood flow in the CA was adequate. We did not apply the MAL division due to concerns regarding stent dislocation induced by dissection procedures near the stent.

OUTCOME AND FOLLOW-UP

Postoperatively, the transaminase level was transiently elevated which quickly improved, and there were no complications related to liver ischemia. No delayed gastric emptying due to decreased gastric blood flow occured, and the patient was able to resume eating four days post-surgery. The patient developed a Grade B postoperative pancreatic fistula (POPF), based on the International Study Group of Pancreatic Surgery grading, but no specific treatment other than drainage was needed[11]. The POPF did not cause any complications, such as hemorrhage and abscess, and the patient was discharged on postoperative day 39. Both the patient and his family were satisfied.

The pathological diagnosis was pancreatic adenocarcinoma, pT1cN0M0, the cancer was StageIA based on the Eighth edition of the UICC-TNM classification, and the pathological treatment effect was graded as IIb based on the Evans classification[12,13]. Since no viable cancer cells were observed in the resected margin, R0 resection was achieved (Figure 5).

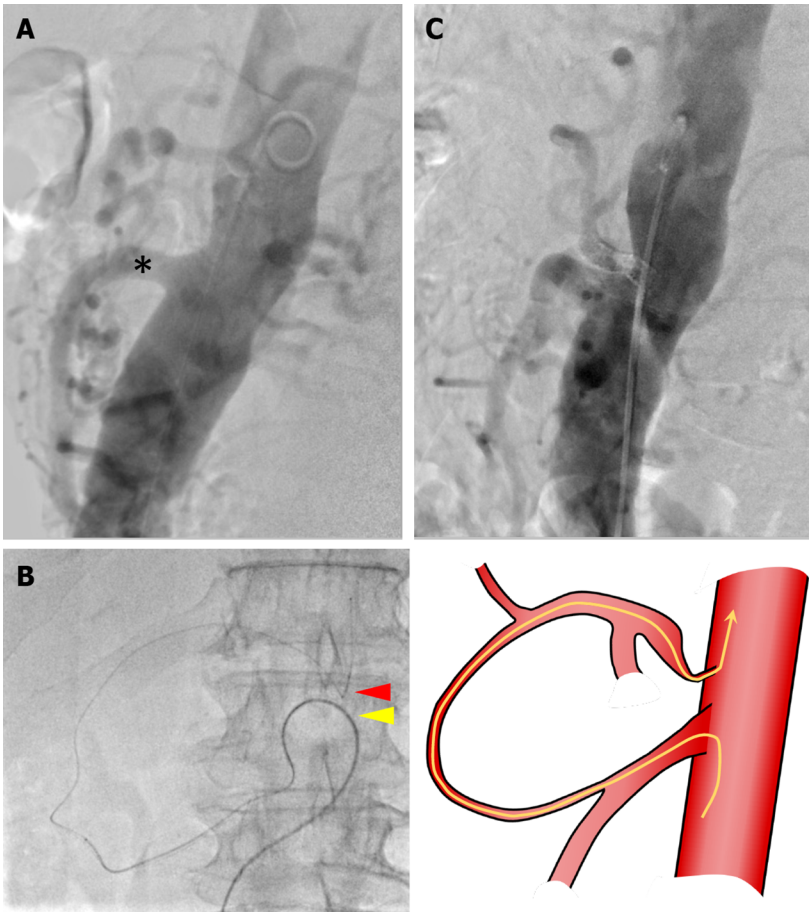


Figure 2 Preoperative endovascular stenting. A: In preoperative aortography, the superior mesenteric artery (SMA) was visualized immediately, but the celiac axis (CA) was not visualized. Black asterisks: SMA; B: The microguidewire reached the CA *via* a collateral pathway from the SMA using a triple coaxial system; C: Final aortography confirmed CA patency and antegrade blood flow. Red arrowhead: Root of the CA; yellow arrowhead: Root of the SMA; yellow line: Running of wire.

Stent patency was confirmed by CT imaging up to six months postoperatively, and the patient had no complications related to liver ischemia (Figure 4) and no recurrence.

DISCUSSION

This case report introduces the procedure and outcome of a preoperative treatment strategy for pancreatic head cancer comorbid with CA stenosis due to compression by the MAL, which combined NAC for cancer and endovascular stenting for MAL compression.

CAS is often observed and reportedly present in approximately 4% to 10.5% of patients undergoing pancreaticoduodenectomy[7,14]. If the GDA is dissected while performing pancreaticoduodenectomy, hepatic blood flow disappears and hepatic ischemia may occur, leading to complications such as liver failure, liver abscess, and bile leakage[1-4]. There are various causes of stenosis, such as MAL compression, atherosclerosis, and arthritis; however, the most common cause is MAL compression[1, 15]. In this case, MAL compression was diagnosed preoperatively, allowing us to intervene preoperatively to maintain hepatic blood flow.

In MAL compression, MAL division is often selected because it is easy and quick. However, while MAL division is simple, it is not reliable, because the CA often does not recanalize and hepatic blood flow is not secured even if the MAL is divided.

Through a comprehensive literature review using the MESH term "pancreat(ic)oduodenectomy and celiac axis stenosis", we identified 108 cases in which the patients underwent pancreaticoduodenectomy and had MAL compression (Table 1). In a literature review of cases in which an intervention was performed for MAL compression, 90.4% underwent MAL division. However, hepatic arterial flow could not be restored in 21.2% of patients despite MAL division. The reason for the failure of MAL division in recanalizing the CA was scarring of the arterial wall caused by longstanding stenosis[16]. In this case, the CT images from approximately 10 years ago already showed signs of MAL compression, which suggested that blood flow might not resume even if the MAL was divided. Additionally, according to a

Table 1 Literature review of previous cases which had median arcuate ligament compression and underwent pancreaticoduodenectomy[24-37]

Factor	Subject	No. of cases (*n* = 108)
Age	yr, median (range)	61 (38-91)[1]
Sex	Male/female	30/19[1]
Diagnosis	Ampullary cancer	4
	Bile duct cancer	10
	Pancreatic head cancer	33
	Others	4
	None described	57
Preoperative detection of CAS	Yes	97
	No	9
	None described	2
Procedure	MAL division	66
	Revascularization	5
	Stenting	1
	Preservation of collateral pathway	1
	No	35
Outcome	Success	91
	Especially MAL division	52
	Failure	17
	Especially MAL division	14
Additional procedures	Revascularization	7
	Stenting	5
	Reoperation	3
	No	3[2]
Complications related to CAS	Liver abscess	3
	Organ ischemia	2
	Anastomotic leakage	3[2]

[1]Among those described.
[2]Indicates some duplication.
CAS: Celiac axis stenosis; MAL: Median arcuate ligament.

previous report which classified MAL compression morphologically by stenosis rate, length of stenosis, and distance between the stenosis and the aorta, these factors were useful in predicting procedures that would be required during the operation. The authors found that MAL division was often ineffective, and revascularization or preservation of the collateral pathways were required, in cases of severe stenosis (stenosis rate of 80% or more) and with a small distance between the stenosis and the aorta[4].

The failure of MAL division can only be determined intraoperatively, and in these situations surgeons have to make a choice that is detrimental to the patient i.e., complex revascularization or abandonment of the surgery. Hepatic arterial revascularization during pancreaticoduodenectomy may result in substantial risk of postoperative hemorrhage – possibly related to POPF – and should be avoided. In previous reports, some cases of CAS had postoperative hemorrhage in the revascularized area[17,18].

NAC has become a common treatment for PC in recent years and is recommended by the NCCN guidelines. NAC is usually administered at an interval of 2-3 mo before radical surgery. With this background, we developed a preoperative strategy to eliminate concerns regarding MAL compression.

In our case, we applied stenting to the CA preoperatively, allowing CA recanalization and hepatic blood flow to be confirmed prior to the operation. Furthermore, DAPT was required to avoid stent

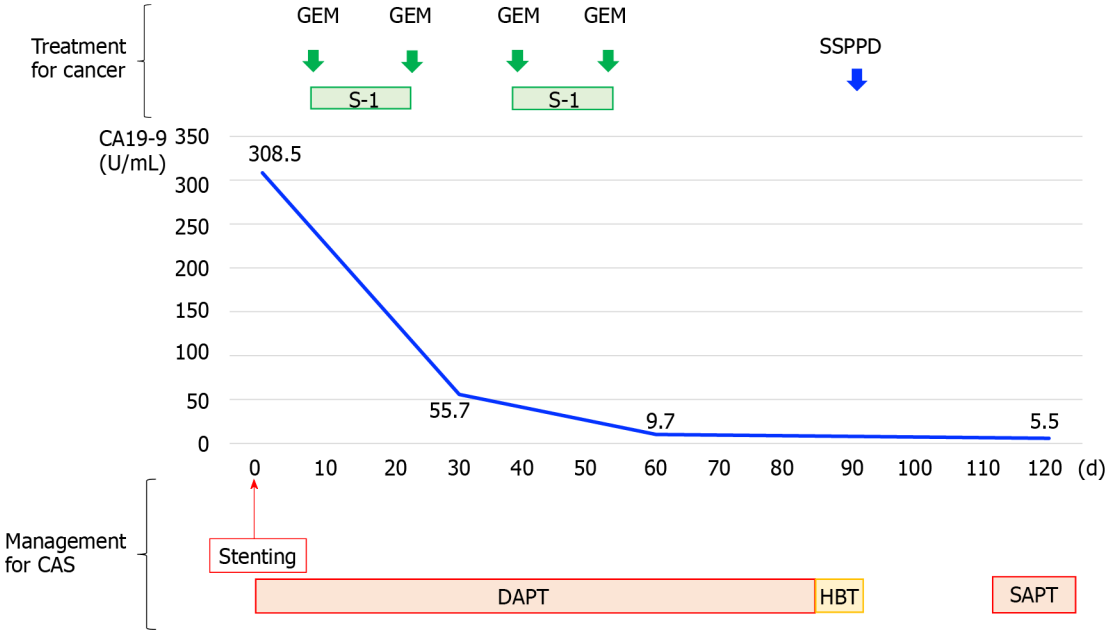


Figure 3 Clinical course timeline. DAPT: Double-antiplatelet therapy; HBT: Heparin-bridging therapy; SAPT: Single-antiplatelet therapy; CA19-9: Carbohydrate antigen 19-9; GEM: Gemcitabine; SSPPD: Subtotal stomach preserving pancreaticoduodenectomy.

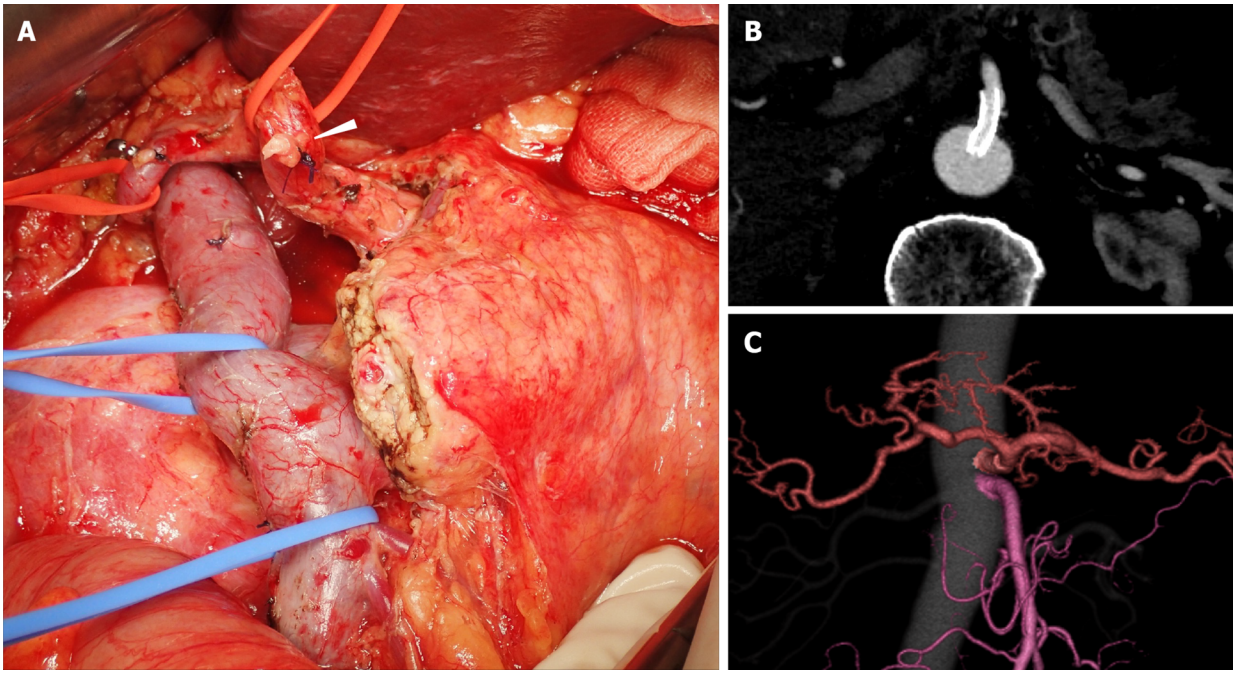


Figure 4 Intraoperative view and postoperative computed tomography images. A: Subtotal stomach preserving pancreaticoduodenectomy was performed. White arrowhead: stump of gastroduodenal artery; B and C: Postoperative computed tomography imaging confirmed patency of the celiac axis.

obstruction, and could also be performed during the NAC treatment period. Clearly, the combination of these two treatments was reasonable. One of the aims in administering NAC is, primarily, to reduce postoperative recurrence. A recent study found that failure of CA19-9 to normalize from preoperatively elevated levels, by the time of surgery, is a predictor of early postoperative recurrence[19]. For our patient, the normalization of the initially very high levels of CA19-9 upon NAC during DAPT, and the preservation of stent patency during the same period, were helpful. The duration of DAPT was determined by referring to the antithrombotic therapy guidelines for patients with coronary artery disease, specifically antithrombotic therapy after percutaneous coronary intervention[20]. MAL division may also be an option to avoid stent obstruction, even after the CA stenting. However, we did not perform MAL division in this case because the risk of dislocating the stent during this procedure defeats the purpose of stenting.

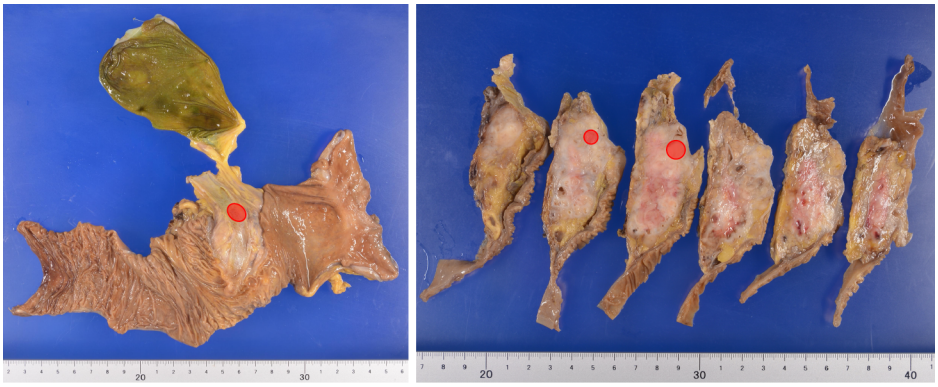


Figure 5 Pathological findings. Tumor mapping on the divided surface of specimens. The resected specimen showed a shrunken invasive tumor with a 12-mm diameter in the pancreatic head. Red circle: Viable tumor site.

Interventional radiology (IVR) for vascular stenosis is performed in various diseases. However, there are few reports regarding the adaptation of IVR for MAL compression, since MAL division is often performed. This case is the first report of the use of the combination of NAC, stent, and DAPT. There is no reason to perform MAL division in addition to this method, and there are no reports of MAL division performed after stenting in existing literature.

Clinical diagnosis of MAL compression is essential for preoperative intervention. In the literature review, a preoperative diagnosis was made in most of the cases (91.5%), indicating that preoperative diagnosis is relatively easy. This high proportion of preoperative diagnosis is thought to be due to the widespread use of multidetector CT and the high recognition of MAL compression[1]. Although the definitive diagnosis of blood flow disturbance by MAL compression can only be made intraoperatively, color doppler ultrasound is a useful diagnostic technique because it is easy and non-invasive[1,3,5].

Intraoperative procedures other than MAL division are used to preserve the collateral pathway, but these surgical techniques are complicated. Additionally, MAL division may impair the curativeness of cancer, so its indications are limited to benign tumors and inflammatory diseases[5,7], and it is not recommended for malignant PC, such as in our case. We were able to dissect the collateral pathway around the SMA and ensure the SMA-margin.

This method has two limitations. First, it requires a high level of skill for stenting in the case of MAL compression. There are previous reports of failure in cases of stenting[21-23]. It is hoped that IVR techniques will become more widespread and improve in the future. Second, the optimal time between stent insertion and radical surgery has not yet been investigated. In this case, since the patient had PC, NAC was administered and sufficient DAPT could be administered during the same period. However, in diseases where NAC is not a common treatment, such as biliary tract cancer, it may worsen the primary disease.

Thus, preoperative simulation and maintenance of skills, such as MAL division and revascularization, remain important for surgeons.

CONCLUSION

Preoperative stent insertion followed by a combination of NAC and DAPT is a safe way to perform pancreaticoduodenectomy; therefore, this procedure may be an effective preoperative treatment strategy.

FOOTNOTES

Author contributions: Yoshida E was the patient's surgeon, reviewed the literature, and contributed to drafting the manuscript; Yoshida E and Kimura Y wrote the paper; Kawagishi R, Sato K, Chiba T, Kimura T, Yonezawa H, and Kobayashi M were involved in the clinical management; Kyuno T, Kono T, and Funato O were assistants at the radical surgery; Murakami K was the radiologist who performed the endovascular stenting; Takagane A and Takemasa I were responsible for the conceptualization and supervision; all authors issued final approval for the version to be submitted.

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Country/Territory of origin: Japan

ORCID number: Eiji Yoshida 0000-0003-1742-3299; Yasutoshi Kimura 0000-0002-7790-9250; Takuro Kyuno 0000-0001-7426-8462; Ryoko Kawagishi 0000-0002-7268-8954; Kei Sato 0000-0002-9812-8912; Tsuyoshi Kono 0000-0001-5549-0594; Takehiro Chiba 0000-0002-2688-5359; Toshimoto Kimura 0000-0002-1972-2295; Hitoshi Yonezawa 0000-0002-6401-4001; Osamu Funato 0000-0003-3353-6971; Makoto Kobayashi 0000-0002-9110-220X; Kenji Murakami 0000-0002-1524-6682; Akinori Takagane 0000-0002-3353-7408; Ichiro Takemasa 0000-0003-1595-2453.

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LETTER TO THE EDITOR

"Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel disease": A letter to the editor

Neasa Mc Gettigan, Aoibhlinn O'Toole, Karen Boland

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Neasa Mc Gettigan, Aoibhlinn O'Toole, Karen Boland, Department of Gastroenterology, Beaumont Hospital, Dublin D09V2N0, Leinster, Ireland

Neasa Mc Gettigan, Aoibhlinn O'Toole, Karen Boland, School of Medicine, Royal College of Surgeons, Dublin D02YN77, Ireland

Corresponding author: Neasa Mc Gettigan, MBChB, MRCP, MSc, Research Fellow, Department of Gastroenterology, Beaumont Hospital, Beaumont Road, Dublin D09V2N0, Leinster, Ireland. neasa13@gmail.com

Abstract

Exercise-induced changes of the microbiome in inflammatory bowel diseases (IBD) is a promising field of research with the potential for personalized exercise regimes as a promising therapeutic adjunct for restoring gut dysbiosis and additionally for regulating immunometabolic pathways in the management of IBD patients. Structured exercise programmes in IBD patients of at least 12 wk duration are more likely to result in disease-altering changes in the gut microbiome and to harness potential anti-inflammatory effects through these changes along with immunometabolic pathways.

Key Words: Inflammatory bowel diseases; Microbiota; Dysbiosis; Metabolism; Exercise; Cytokines

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Core Tip: Exercise-induced changes of the microbiome in inflammatory bowel diseases (IBD) is a promising field of research with the potential for personalized exercise regimes as a promising therapeutic adjunct for restoring gut dysbiosis and additionally for regulating immunometabolic pathways in the management of IBD patients. We have observed that exercise programmes of at least 12 wk duration are required to exert any meaningful effects on gut dysbiosis restoration and suggest that the positive effects of a more prolonged programme may extend to inflammatory mediation through regulation of immunometabolism.

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TO THE EDITOR

We read with interest a review article by Koutouratsas *et al*[1] on the "Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel diseases: A review".

We agree with the authors conclusion that the effects of prescribed exercise on the microbiome is a promising area for further research and that the potential for personalized exercise regimes is a promising therapeutic adjunct when considering the restoration of gut dysbiosis in the management of inflammatory bowel diseases (IBD) patients.

With personalization of exercise regimes in mind, we find it is pertinent to consider the duration of any given exercise programme prescribed for IBD patients. This review article presents the findings of a number of clinical trials in humans examining the effect of various forms of exercise on gut microbiome composition, functionality and diversity. Interestingly, we would like to remark on the duration of exercise programmes and to highlight that the studies of short-term exercise programmes (6 wk duration or less) did not show any clinically significant effect on gut microbiome diversity or composition[2,3] in comparison to studies of at least 12-wk duration which showed changes in gut microbiome composition, diversity and functionality[4,5]. A study of IBD patients not included in the review of 8 wk duration of a prescribed aerobic exercise programme also did not show any significant difference in gut microbiome composition/diversity in response to the exercise programme but other benefits were demonstrated including an improvement in muscle mass and body fat %[6]. Furthermore, two studies of elite athletes, one of rugby players and the other of rowers showed significant differences in microbiome with exercise which likely reflects the habitual nature of the exercise in addition to other factors such as diet[7,8].

A range of exercises have been shown to be safe in patients with IBD including moderate intensity aerobic exercise, resistance training and high intensity interval training[6,9,10]. We suggest that any future studies examining the effects of exercise on changes in the gut microbiome should be of at least 12 wk duration with consideration given to the recommended physical activity guidelines to avoid potential harmful effects of excessive vigorous exercise whilst also being mindful of disease activity (*i.e.*, a personalized approach would be the optimum)[11-13].

Exercise has been shown as a promising therapeutic intervention or adjunct to influence metabolism in disorders including multiple sclerosis through regulation of immune cells[14]. This is mediated through cytokine secretion, and modulation of metabolic regulators including tryptophan[15,16]. Therefore, we suggest that future studies on the effects of structured exercise programmes in IBD patients should be at least 12 wk duration to promote disease-altering changes in the gut microbiome and harness potential anti-inflammatory effects through these changes along with immunometabolic pathways. These benefits would be in addition to promoting sustained exercise behavioral patterns.

FOOTNOTES

Author contributions: Mc Gettigan N wrote the letter, O'Toole A and Boland K revised the letter.

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Country/Territory of origin: Ireland

ORCID number: Neasa Mc Gettigan 0000-0001-5744-0435; Aoibhlinn O'Toole 0000-0002-1426-2787; Karen Boland 0000-0003-2327-9924.

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