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MINIREVIEWS

Nanomaterials: Applications in the diagnosis and treatment of pancreatic cancer

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Abstract

Pancreatic cancer (PC) remains one of the leading causes of cancer-related death in human sowing to missed early and effective diagnosis. The inability to translate research into clinical trials and to target chemotherapy drugs to tumors is a major obstacle in PC treatment. Compared with traditional cancer detection methods, the method combining existing clinical diagnosis and detection systems with nanoscale components using novel nanomaterials shows higher sensitivity and specificity. Nanomaterials can interact with biological systems to efficiently and accurately detect and monitor biological events during diagnosis and treatment. With the advance of experimental and engineering technology, more nanomaterials will begin the transition to clinical trials for their validation. This paper describes a number of nanomaterials used in the diagnosis and treatment of PC.

Key words: Nanomaterials; Pancreatic cancer; Diagnosis; Treatment; Nanoparticles; Quantum dots

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Core tip: Pancreatic cancer remains one of the leading causes of cancer-related death in humans owing to missed early and effective diagnosis. Nanomaterials can interact with biological systems to efficiently and accurately detect and monitor biological events during diagnosis and treatment in pancreatic cancer.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most common malignancies of the digestive system. It is a highly fatal disease with a 5-year survival rate of less than 5%[1]. PC is highly aggressive and can easily invade adjacent tissues and metastasize to distant organs[2,3].Additionally, PC is significantly resistant to standard chemotherapy, so the most effective treatment for PC is surgical resection[4,5]. About 80% of PC patients are diagnosed at an advanced stage because of the lack of clinical symptoms at early stages of the disease. Surgical treatment at these late stages is suboptimal because of the high risk of metastasis[6]. Computerized tomography and endoscopic ultrasonography with or without fine needle biopsy have been widely used for diagnosing and staging of PC and computerized tomography with pancreas protocol and endoscopic ultrasonography correlate moderately well in terms of mass detection, mass size, vascular involvement and lymph node involvement[7,8]. However, the two methods also have some drawbacks. The diagnosis results are related to the clinician's ability to read the image and the proficiency of endoscopic operation, which may lead to missed diagnosis. Therefore, a new method for early detection and diagnosis of PC is urgently needed.

The rapid development of new nanomaterials in recent years may lead to improvements in the diagnosis of PC and therefore benefit patients. In many tumor models, nanomaterials have been proved to be effective, and it is expected that these nanomaterials will be more widely applied to clinical applications in the future. Compared with traditional biosensor systems, nanomaterials have many advantages and great development potential. Using nanoscale components to construct nanomaterials can greatly improve detection sensitivity. In addition, by expanding the upper and lower limits of biomarker detection, the indications for surgical treatment are effectively expanded. Furthermore, advances in manufacturing technology can reduce the cost of producing these nanosensors, thereby reducing the medical costs of detecting and diagnosing cancer and reducing the burden on patients[9]. Recently, much academic research has been devoted to the testing and development of nanotechnology, which promises to improve the detection of existing diseases.

Nanomaterials work at the nanoscale, which is broadly defined as 1 to 500 nm, or about a billionth of a meter. Through multidisciplinary cooperation in the fields of medicine, biology, chemistry, physics, engineering and technology, the development of nanomaterials has achieved great success[10]. There are two main reasons for the characteristic properties of nanomaterials at such a small size. First, there is a large surface area to volume ratio, and many of the atoms that make up the material are very close to the surface. Second, since the size of the material is close to the wavelength that excites the components of the nanomaterial, it exhibits quantum forces[11]. This review will introduce the characteristics of some nanomaterials and their applications in the diagnosis or treatment of PC.

NANO BIOMATERIALS

Nanoparticles

To date, carbohydrate antigen (Ca) 19.9 is the only PDAC marker approved by the United States Food and Drug Administration. However, its use is discouraged in the diagnostic phase because of the poor sensitivity (60%-70%) and specificity (70%-85%)[12]. In many nanobiomaterials systems, nanoparticles (NP) are often key components in detecting cancers. These particles are made of a variety of materials, and each of them has unique properties which are designed specifically to enhance the ability to detect biomarkers. With advances in nanotechnology and our understanding of materials at the nanoscale, nanoparticles have been approved or entered clinical application, including diagnostic applications, such as imaging or biomarker testing, as well as therapeutic applications, or a combination of diagnostic and therapeutic applications, also known as therapeutic diagnostics[13].

Quantum dots

Quantum dots (QDs) are a class of nanoparticles used to detect cancer *in vitro* and *in vivo*. These nanocrystals are composed of semiconductor particles with inorganic elements at their core and surrounded by metal shells[14]. They are generally less than ten nanometers in diameter[15] and the strengths of quantum dots in cancer diagnosis and treatment and even the application in cancer study stem from their unique characteristics. The first is the ability to adjust the size and composition of quantum dots so that they have unique fluorescence excitation wavelengths, ranging from 400

nm to 2000 nm[16]. QDs can be tuned to any color to accommodate different wavelengths, making it possible to identify and track different biomarkers by applying only one single light source. In addition, the other one useful feature of quantum dots is that they are reusable and have a long lifespan, possibly due to their resistance to fading[17]. One problem with normal healthy tissue imaging is that it often exhibits autofluorescence, which interferes with signals coming from tissue in cancer[13]. Quantum dots are designed to combine with fluorescent properties in the near-infrared spectrum, so the autofluorescence interference can be eliminated[14]. One potential problem with using QDs *in vivo* is the risk of toxicity after injection. Some improvements have been made to reduce the potential toxicity, but more studies are needed to determine the appropriate clinical solutions.

Carbon nanotubes

Carbon nanotubes (CNT) are hexagonal structures composed of self-aligned single-walled or double-walled carbon molecules, with diameters ranging from 0.3 nm to 100 nm[18]. CNT have several properties that make them more suitable for use in nanomaterials. The characteristics of resistance to high heat and electrical conductivity permit CNT to function normally even when exposed to high temperatures or electrical currents. CNT also show high-tensile strength, making them resistant to permanent deformation due to physical forces. In general, CNT can be divided into two categories due to the different principles in recognizing biomarkers: one is based on electrochemical signals generated by redox reactions, and the other is based on detection signals of field effect transistors generated by surface charges of CNT[19]. In the first category, both single-walled and multi-walled CNT are used in a type of application called a nanotube forest. The technique requires the nanotubes to be lined up in parallel so that antibodies to specific biomarkers are attached to the surface. The second type of detection is the aforementioned field effect transistors method, in which nanotubes are connected to electrodes at either end by lithography and functionalized to bind biomarkers. Once these biomarkers are bound, a decrease in the conductivity of the nanotubes is detected, and is proportional to the amount of binding.

Gold nanoparticles

Gold nanoparticles make up another class of promising particles in diagnostics and are made from colloidal gold. These nanoparticles exist mainly in the form of gold nanospheres and exhibit a strong ruby color in aqueous solution. The intriguing optical properties of gold nanoparticles are due to local surface plasmon resonance, in which the valence electrons of gold nanoparticles oscillate with incident light at a specific frequency[20]. Some of the energy absorbed by gold nanoparticles is emitted as scattered light, which forms the basis of optical imaging of gold nanoparticles. The rest of the energy decays in a non-radioactive form, turning into heat that can be used to kill cancer cells, thus acting as photothermal therapy[21]. Gold nanoparticles have been found to be widely used in multiple potential tumor diagnosis and treatment fields.

Liposomes

Liposome, which can be applied to transport a drug to the location of tumors, is one type of nanoparticle consist of a lipid bilayer[22]. Like other types of nanoparticles, liposomes rely on enhanced permeability and retention (EPR effects) to enter tumor blood vessels and stay close to the tumor. The phenomenon of EPR stems from the structural characteristics of the vascular system in solid tumors. Nanoparticles increase the ability to exude through less tight endothelial connections. Once in the tumor microenvironment, the retention of nanoparticles increases due to inadequate lymphatic drainage. To take advantage of the EPR effect, liposomes are typically designed to be less than 200 nm in size to improve the amount of retention in the tumor[23]. Liposomes are able to be targeted with unique transport components to increase cellular uptake in the target tissue.

NANO BIOMATERIALS AND PC

Nanoparticles and PC

One type of nanoparticle used to target PC cells was organically modified silica nanoparticles with a diameter of about 20 nm. The surface of these nanoparticles was functionalized with transferrin, stabilizer four and antimycin, and the fluorophore rhodamine B was used as the imaging agent. This technique is simple to operate and suitable for photobiological imaging research[24]. Similarly, another study indicated that mesoporous silica nanospheres functionalized with the target ligand and were

covalently attached with a rare earth ion Gd(III) *via* a disulfide group. *In vitro*, that was shown to be an effective MRI contrast agent in models with PC. However, the disulfide was rapidly metabolized *in vivo*[25].

In addition, parvifloron D (PvD), a natural compound isolated from the Plectranthus genus, showed cytotoxicity and anti-proliferation activity on human leukemic cancer cells. However, PvD is a compound with very low water solubility, and nanotechnology was an effective strategy to solve this problem using albumin nanoparticles prepared by desorption. The nanoencapsulated PvD showed selective cytotoxicity towards PC cell lines. Further optimization that included glucose crosslinking resulted in PvD that was wrapped in nanoparticles that were molded to a similar size (100–400 nm) and shape. The coupling of erlotinib to these PvD nanoparticles, led to substantial antiproliferative activity in the BxPC-3 PC cell line[26].

Gemcitabine (GEM) is a first-line, standalone chemotherapy drug for PC, but tumor cells develop resistance to it after a few months. Multiple clinical and preclinical studies have shown that GEM in combination with other chemotherapy drugs can achieve better therapeutic results. However, this type of combination therapy often causes severe systemic toxicity. Hence, new treatment regimens need to be developed to safely deliver chemotherapeutic drug combinations to patients. Nanoparticles mediate drug delivery by incorporating drug combinations at the same time, similar to a "cocktail of drugs", targeted to tumor cells[27].

Quantum dots and PC

Noninvasive acquisition of tumorigenesis and metastasis information requires nontoxic, long-acting fluorescent probes for tumor imaging. A biocompatible near infrared ray (NIR) fluorescent probe modified with glucose (Glc) on the surface of NIR Ag2Se quantum dots (NIR Ag2Se QDs) has been used to image tumors *in vivo*. These glucose-functionalized Ag2Se QDs (Glc-Ag2Se QDs) were observed *in vivo* for at least 7 d. In addition, the probe was excreted through the kidney, and the excretion capacity is conducive to the application of imaging *in vivo*. Furthermore, Glc-Ag2Se QDs were used for targeted imaging of human breast cancer cells (MCF-7) as well as imaging of SW1990 PC cells[28]. Detection of circulating tumor cells (CTCs) play a vital role in the diagnosis of tumors. CTCs disseminate from the primary tumor using the circulatory or lymphatic system and can form secondary tumor colonies. The number of CTCs has been used as an indicator of cancer progression. However, the population of CTCs is very heterogeneous and the identification of CTC subsets such as tumor stem cells with high metastatic potential is a very challenging task but has great importance for the diagnosis and management of tumors. With the help of quantum dots, CTCs with higher metastatic potential, such as CD24+ and CD133+ CTCs, have been identified in live animals and may reveal detailed mechanisms of metastasis, such as tumor cell extravasation into blood vessels[29]. ZnO QDs are also used for the detection of CA19-9 cancer biomarkers using an immunosandwich method, involving square wave extraction voltammetry and photoluminescence, to enable better detection of PC cells[30].

CNT and PC

CA19-9 is an important tumor antigen of pancreatic, bile duct, stomach, colorectal and liver cancer. CA19-9 has been used to construct a novel immune sensor consisting of CNT, gold nanoparticles and SiO_2 nanoparticles. The process used to make the nanosensor involves the outer surface of the CNT being covered with bovine serum albumin molecules. Gold nanoparticles are then attached to bovine serum albumin-CNT, and the electrochemical deposition of gold provides nucleation sites for the first gold nanoparticles. These initial steps provide a large surface where the CA19-9 antibody can be immobilized and used as a sensing component. To improve the signal and the sensitivity of detection, SiO_2 nanoparticles are modified as secondary antibodies for a sandwich immunoassay. Experiments at different concentrations of CA19-9, showed a detection threshold 100 times lower than the ELISA standard currently applied in clinical practice, and therefore could be more effective in diagnosing early PC[31]. Multi-walled CNTs have also been used to build genetic fingerprints of PC. Studies have shown that multiwalled CNT electrochemical sensing were combined with random amplified polymorphic DNA. This detected differences in guanine and deoxyguanine triphosphate between DNA samples in peripheral blood from PC patients and control patients[32].

Gold nanoparticles and PC

A recent study suggested that a drug delivery nanosystem based on gold nanoparticles (PEGAuNPs) was able to effectively deliver drugs to tumor cells. Adriamycin and volitinib an anthracycline and tyrosine kinase inhibitor respectively, were coupled with gold nanoparticles and modified for size, stability and

morphology. The combination of PEGAuNPs with adriamycin and varlitinib proved effective in human cells, with PEGAuNPs further suppressing cancer cell proliferation and reducing the toxicity to normal cells[33]. Another study by Banstola *et al*[34] indicated that chemotherapeutic photothermal combination therapy could improve the efficacy of chemotherapeutic drugs in patients with PC. Specifically, the combination of paclitaxel-targeted polydopamine polymer microspheres and controllable gold nanoparticles suggested significant improvement in the efficacy of photothermal chemotherapy in PC cell lines[35].

Liposomes and PC

Extracellular matrix (ECM) overexpression in pancreatic ductal adenocarcinoma cells limits drug penetration into tumors and is associated with poor prognosis. A recent study demonstrated that a proteolytic enzyme, which underwent pretreatment based on the nanoparticle system, decomposed the dense extracellular collagen matrix of PC and increased the penetration of drugs in an orthotopic murine PC model. More specifically, this collagenase was a proteolytic enzyme encapsulated by a 100-nanometer liposome, which was designed to protect collagenase from premature inactivation, prolonging its release rate at the target site, and effectively improving the efficacy of the drug[34]. Interestingly, degradation of the extracellular matrix did not increase the number of circulating tumor cells or metastases. This strategy also has the potential to degrade the extracellular matrix in other diseases, such as liver fibrosis, and improve tissue permeability before administration[36]. Similarly, the effect of gemcitabine in pancreatic ductal adenocarcinoma is limited by dense fibrosis, as well as pharmacokinetics and low blood flow. To address this, activated liposomes that release drugs through local heating may enhance serum stability and circulatory effects; the released drugs also retain the ability to spread within the tumor. In a mouse model of more aggressive PC, local hyperthermia with liposomes combined with gemcitabine induced cell death and the formation of apoptotic areas[37]. The summary was showed in Table 1.

CONCLUSION

This article reviews some of the current developments in nanomaterials for the diagnosis and treatment of PC. The efficacy of nanomaterials depends on following factors, including targeted delivery of nanoparticles to tumor sites, EPR effects, tumor heterogeneity, tumor microenvironment and metastasis prevention, nanobiological interactions, and host-tumor immune crosstalk. The synthesis of nanomaterials is also important for the diagnosis and treatment of PC. With continued research and development efforts we expect targeted NPs to have a tremendous impact on human health for decades to come.

Table 1 The summary of the application of nanomaterials in pancreatic cancer in this study

Nanomaterials	Example	Function
Nanoparticles (NPs)	Silica NPs	Photobiological imaging research[24] MRI contrast agent[25]
	Albumin NPs	Wrap anti-proliferation drug (PvD) to target cancer cell[26]
	Delivery NPs	Safely deliver Gemcitabine to tumor cells and eliminate toxic effects on normal cells[27]
Quantum dots (QDs)	Ag2Se QDs	Image pancreatic cancer (PC) *in vivo*[28]
	CTC QDs	Identify CD24+ and CD133+ to detect CTCs[29]
	ZnO QDs	Better detection of CA19-9 and PC cell[30]
Carbon nanotubes (CNTs)	CA19-9 CNTs	Construct a novel immune sensor and more efficient detection of early PC[31]
	Multi-walled CNTs	Built genetic fingerprints of PC[32]
Gold nanoparticles	PEGAuNPs	Effectively deliver drugs to tumor cells
		Further suppressing cancer cell proliferation and reducing the toxicity to normal cells[33]
		Improvement in the efficacy of photothermal chemotherapy in PC[34]
Liposome	Collagenase liposome	Prolonging drug release rate at the target site, and effectively improving the efficacy of the drug[35]
	Gemcitabine liposome	Local hyperthermia with liposomes combined with gemcitabine induce cell death and the formation of apoptotic areas[37]

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REVIEW

Management of gastric outlet obstruction: Focusing on endoscopic approach

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Abstract

Gastric outlet obstruction (GOO) is a medical condition characterized by epigastric pain and postprandial vomiting due to mechanical obstruction. The obstructions typically involved in GOO can be benign or malignant. Peptic ulcer disease is the most common cause of benign GOO, and malignant causes include gastric cancer, lymphoma, and gastrointestinal stromal tumor. With the eradication of *Helicobacter pylori (H. pylori)* and the use of proton pump inhibitors, the predominant causes have changed from benign to malignant diseases. Treatment of GOO depends on the underlying cause: Proton pump inhibitors, *H. pylori* eradication, endoscopic treatments including balloon dilatation or the placement of self-expandable stents, or surgery.

Key words: Gastric outlet obstruction; Balloon dilation; Metal stent

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Core tip: The causes of gastric outlet obstruction are generally divided into benign and malignant. With the eradication of *Helicobacter pylori* and the use of proton pump inhibitors, the predominant causes have changed from benign to malignant diseases. Treatment of gastric outlet obstruction (GOO) depends on the underlying cause: Proton pump inhibitor, endoscopic techniques, or surgery. In this article, we review the etiology, diagnosis, and current treatment methods of GOO, especially endoscopic techniques.

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INTRODUCTION

Gastric outlet obstruction (GOO) occurs when gastric emptying is mechanically inhibited by various diseases, most of which involve obstruction of the gastric pylorus or proximal duodenum due to intrinsic or extrinsic factors. The precise incidence of GOO is unknown. Although GOO due to peptic ulcers has been common in the past, the use of proton pump inhibitors and identification of *Helicobacter pylori (H. pylori)* have reduced the incidence of peptic ulcer disease, and malignant diseases have become the main cause of GOO in recent decades, with about 50%-80% of GOO being caused by cancer[1-5]. As the predominant cause of GOO shifts from benign to malignant diseases, treatment methods have also changed. In this article, we review the etiology, diagnosis, and current treatment methods of GOO, especially endoscopic techniques.

ETIOLOGY

Benign gastric outlet obstruction

Peptic ulcer disease is the most common cause of benign GOO, accounting for approximately 90% of cases[6]. Caustic ingestion, inflammatory diseases such as Crohn's disease or tuberculosis, and non-steroidal anti-inflammatory drug-induced strictures may also result in GOO. Other rare benign causes are large gastric polyps, gallstone obstruction (Bouveret's syndrome), annular pancreas, pancreatic pseudocyst, and bezoars (Table 1)[7]. Peptic ulcer disease was the leading cause of GOO in the past, with the use of proton pump inhibitors and identification of *H. pylori*, the incidence has declined significantly. Currently, GOO is the least common complication of peptic ulcer disease. Less than 5% of complicated duodenal ulcer disease and less than 1-2% of complicated gastric ulcer disease develop obstructive complications[8,9].

Corrosive injury caused by caustic ingestion, including acid or alkali substances, can result in GOO by antral or pyloric scarring[10,11]. The incidence rate of GOO by caustic ingestion varies from 20 to 60%[10-12]. In a study of 41 cases of acid ingestion, 44.4% developed GOO[10], and another study reported that 36.8% of the 31 alkali-ingestion patients developed GOO[11].

Inflammatory causes, such as Crohn's disease or tuberculosis, also cause GOO. Crohn's disease mostly invades the distal gastrointestinal tract, and it rarely invades the upper gastrointestinal tract such as the stomach or duodenum alone. Clinically, severe gastroduodenal Crohn's disease is rare, in which case it continuously invades the antrum, pylorus, and proximal duodenum[13]. Gastroduodenal tuberculosis is rare, occurring in only 0.3%-2.3% of patients with tuberculosis. GOO was identified in 61% of 23 patients with gastroduodenal tuberculosis which was confirmed by histopathological examination[14].

Nonsteroidal anti-inflammatory drugs (NSAIDs) can also cause GOO. NSAIDs reduce prostaglandin E2 to induce pyloric edema and scarring, and increase histamine release to increase gastric secretion, reduce mucosal absorption, and cause gastric motility disturbances, leading to GOO[15]. In a study of 10 cases of NSAID-induced GOO in 2011, the most common site of involvement was the duodenum, followed by the pylorus and duodenum, and then pylorus only[16]. Most strictures were short web-like in nature, and endoscopic balloon dilation was successful in 90% of cases.

Malignant gastric outlet obstruction

In recent decades, malignancy has been the most common cause of GOO. The most common causes are pancreatic and gastric cancer, but lymphomas, duodenal carcinoma, biliary tract carcinoma, ampullary carcinoma, and metastatic malignancies can also cause malignant GOO. In pancreatic cancer, 15%-20% of patients have been reported to develop GOO[17].

DIAGNOSIS

The diagnosis of gastric outlet obstruction is usually suggested by history and physical examination. Patients have suffered recurrent vomiting and show up electrolyte abnormalities including hypokalemia or hypochloremic metabolic alkalosis. The gastrin secretion due to gastric expansion increases serum gastrin levels (400-800 pg/mL range) and can be confused with Zollinger-Ellison syndrome[18]. Tests such as endoscopy, and barium study are helpful for diagnosis. Plain radiography

Table 1 Causes of gastric outlet obstruction

Benign	Malignant
Peptic ulcer disease	Gastric cancer
Caustic ingestion	Gastric lymphoma
NSAID induced stricture	Pancreas cancer
Bouveret syndrome	Duodenal cancer
Hypertrophic pyloric stenosis	Cholangiocarcinoma
Iatrogenic	Gallbladder cancer
Post-surgical scar or anastomosis stricture	Metastatic cancer
Endoscopic submucosal dissection	
Endoscopic mucosal resection	
Inflammatory causes	
Crohn's disease	
Pancreatitis	
Inflammatory polyps	
Infectious causes	
Tuberculosis gastroenteritis	
CMV gastroenteritis	
Infiltrative causes	
Eosinophilic gastroenteritis	
Amyloidosis	

NSAID: Nonsteroidal anti-inflammatory drugs; CMV: Cytomegalovirus.

may show a large gastric shadow. Contrast studies with barium or water-soluble contrast agents may show an enlarged stomach and provide clues as to the underlying disease. The absence of any contrast passage in the small intestine suggests a complete GOO. CT scan is helpful, especially for evaluating the mural thickness of the pylorus or gastric wall, lymph node enlargement, pancreatic or biliary tract, and retroperitoneum[9].

Endoscopy is the most useful examination to establish gastric outlet obstruction and obtain tissue specimens from obstructing areas for confirmation or exclusion of malignant GOO. Endoscopy should be performed after fasting for over 4 hours, and nasogastric tube suction is recommended before endoscopy to reduce the risk of aspiration.

TREATMENT

All patients with symptomatic GOO need to be hospitalized. Fluid resuscitation with normal saline and correction of electrolyte imbalance should be performed first. Nasogastric decompression should be initiated during hospitalization. This helps relieve discomfort and pain caused by gastric distension, clear the field during endoscopic procedure, and reduce the gastric capacity before surgery. In patients with benign gastric outlet obstruction due to acute peptic ulcer disease, patients showed improvement in symptoms due to reduced edema and spasm due to inflammation after 48-72 h with nasogastric decompression and proton pump inhibitors.

H. pylori eradication can be performed in patients with benign GOO with *H. pylori* infection. The prevalence of *H. pylori* in GOO varies from 33% to 90%[19]. Kate *et al*[20] reported a high prevalence of *H. pylori* infection in duodenal ulcers with GOO, even without active ulcers. Acute ulcers associated with *H. pylori* infection cause obstruction due to inflammation and edema, and antimicrobial treatment can help improve occlusion. Mohsina *et al*[21] summarized reports on the role of *H. pylori* in GOO.

If GOO is irreversible with medical therapy, definitive treatment is required based upon the underlying cause (Table 2). Until the development of endoscopic procedures, surgery was the only treatment for these patients. In the past, 80%-90% of ulcer related GOO patients underwent surgery[22], and the only treatment option for caustic GOO patients was surgery as well[12]. Recent reports suggest that endoscopic balloon dilation is an effective treatment option, as an alternative to surgery in the

majority of peptic ulcer disease-related and caustic GOO patients[23-31]. In benign GOO, intraluminal stent insertion is a poor treatment option. There are no commercial stents available for benign GOO, and if uncovered stents are used, stent removal is impossible, and long-term patency is not guaranteed, and stent migration occurs frequently when covered stents are used. On the other hand, if curative surgery is not possible in malignant GOO, there are palliative options such as endoscopic placement of self-expanding metal stents (SEMS), and bypass surgery such as gastrojejunostomy. Surgical gastrojejunostomy for palliative purposes has a high mortality of up to 10%[32], and previous reports have shown that palliative SEMS insertion is more cost-effective, reduces the number of days of hospitalization, and improves symptoms rapidly[33,34]. Endoscopic SEMS insertion is widely performed in malignant GOO.

ENDOSCOPIC MANAGEMENT

Endoscopic balloon dilation

Benjamin *et al*[35,36] first reported the use of endoscopic balloon dilation (EBD) of the pylorus for the treatment of GOO using a through-the-scope 5-mm balloon with good clinical outcome. Subsequent reports have shown the safety and effectiveness of EBD for GOO management[23-31]. Dilations can be performed with endoscopy and using balloon dilators inserted through the working channel of the endoscope, or using balloons placed over a guidewire under fluoroscopic guidance. If adequate dilation is achieved, the clinical response is maintained in 70%-80% of patients[25,30]. Repeated recurrence of stricture after EBD may be an indication of surgery. If more than two sessions of dilations are required, they are highly associated with the probability of surgery[28].

EBD may also be effective for GOO caused by caustic injury or endoscopic submucosal pylorus dissection[37,38]. In a single-center study published by Kochhar *et al*[39] recently, EBD had a clinical success of 97.3% and no recurrence during a 98-month follow-up period. Perforation occurred in 2 of 111 patients. However, the mean number of sessions was 2-13 times in caustic GOO, while only 1-3 times in PUD-induced GOO[27,38]. GOO caused by other causes, such as Crohn's disease and tuberculosis, may also benefit with EBD[26,40].

EBD is generally a safe procedure, with complications of bleeding and perforation in diameters less than 15 mm rare. Perforation occurred more often when the diameter was over 15 mm[7,24,41,42]. Pain and minor bleeding are common during EBD procedures, but they are self-limited, whereas arterial bleeding is rarely reported[40].

Intralesional steroids

A combination of balloon dilation and intralesional steroid injection could be performed to inhibit stricture formation. Triamcinolone blocks the cross-linking of collagen and prevents scar contracture[43]. There are few reports on the treatment of pyloric strictures with intralesional steroids. Kochhar *et al*[44] and Lee *et al*[45] reported the efficacy of intralesional steroids.

Endoscopic incision

Endoscopic incision could be further performed after endoscopic balloon dilation in pyloric stenosis refractory to EBD. Boron *et al*[46] reported an electrosurgical incision using sphincterotomy, and Hagiwara *et al*[47] used a needle-knife radial electrosurgical incision in refractory anastomotic pyloric stenosis.

Endoscopic placement of self-expanding metal stents

SEMS insertion is used as a palliative treatment for malignant GOO and is used in cases of malignant gastrointestinal obstruction that cannot be surgically treated. The goal of SEMS insertion is to relieve obstruction symptoms. To evaluate the degree of symptom relief, the GOO score, which evaluates the severity of symptoms defined as satiety, nausea, and early vomiting, scoring based on the patient's oral intake level[48].

Generally, the technical and clinical success rates are reported to be 89%-98% and 86%-89%, respectively, which is very good in terms of short-term success rates[49-52]. SEMS insertions should be considered in patients with a short life expectancy (less than 2-6 mo)[53]. In addition, there should be no other occlusion site in the distal part of the stent insertion site, and the presence of free perforation or peritonitis are contraindications to endoscopic stent placement[54].

In malignant GOOs, biliary obstruction is often coexistent. Placement of the biliary metal stent should be considered before insertion of the duodenal stent. Since the endoscopic approach to the biliary tract is very limited after the duodenal stent is inserted, the percutaneous transhepatic approach is usually required[54].

Table 2 Treatment of gastric outlet obstruction based upon the underlying cause

Underlying cause	Treatment	
Benign		
Peptic ulcer disease	PPI ± HPE (1st option)	EBD or surgery (2nd option)
Crohn disease	Corticosteroid (1st option)	EBD or surgery (2nd option)
Caustic ingestion	EBD or surgery	
Bouveret syndrome	Surgery or endoscopic removal	
Large gastric polyp	Endoscopic resection	
Malignant		
Palliative	Endoscopic stent (covered or uncovered)	
	EUS-guided gastroenterostomy	
	Surgical resection, surgical bypass (gastrojejunostomy)	
	Radiation therapy	
Curative	Surgery	
	Chemotherapy (for lymphoma)	

PPI: Proton pump inhibitor; HPE: Helicobacter pylori eradication; EBD: Endoscopic balloon dilatation; EUS: Endoscopic ultrasound.

Covered versus uncovered SEMS: Uncovered stents are widely used for the treatment of malignant GOO. It is less likely to migrate and more flexible, but the tumor can grow into the stent and result in stent obstruction. Covered stents are increasingly used in Europe because they provide the advantage of low tumor growth. However, they are more prone to migration and less flexible than uncovered stents[55,56]. According to Kim *et al*[57] stent migration rate was much higher in covered stents than in uncovered stents (28% *vs* 3%) within 8 weeks of stent insertion.

According to a systematic review by Yang *et al*[58], there were no significant differences in technical or clinical success rate, long-term patency, or complications in three meta-analyses, in which comparison of efficacy and safety between covered or uncovered SEMS for malignant GOO were assessed.

Currently uncovered SEMS, rather than fully or partially covered stents, have been shown to be a standard treatment for managing malignant GOO, with low migration rates and better bile outflow[55,56,59]. Tumor ingrowth/overgrowth has been reported in 17.2% of patients receiving bare metal stents and in 6.9% of patients with covered stents[60,61]. This stent obstruction can be managed with a stent-in-stent technique, and stent occlusion rate was reported to be 10%-34% after the secondary SEMS insertion[62,63]. The development of stents to compensate for the shortcomings of the existing stents continues, and recent new covered stents with anti-migration designs have been suggested to be superior in terms of stent patency and complications[64].

SEMS *vs* surgery: The comparison of the effects and safety of surgical methods and endoscopic stents as palliative treatment for malignant GOO have been presented in various studies. Compared to surgery, the advantages of endoscopic stents are; shorter procedure time, less time to ingestion, and shorter hospitalization periods, but repeated procedures are often required due to frequent stent failures[65-67]. According to one systematic study, patients treated with enteral stents showed shorter hospitalization periods (average 12 d) and faster oral intake (average 7 d) than those treated with gastrojejunostomy, and there was no significant difference in mortality, overall complications, and survival rates[68]. Most studies have shown that there is no difference between both treatments in technical or clinical success rate of the procedure, but one meta-analysis reported that the success rate was higher in stent placement patients[69,70]. There was no difference in the frequency of mild and severe complications in the early stage of complications after SEMS insertion or surgery, but it is known that the time of severe complications in the late stage is relatively earlier and more common in stent patients[71]. Nevertheless, there was no difference in stent insertion or surgery-related mortality[72]. In larger randomized trials with longer follow-up, late complications including recurrent obstruction and need for reoperation were more common in SEMS than gastrojejunostomy, which confirms the previous retrospective study, which reported that gastrojejunostomy surgery has more benefits and is associated with a longer life expectancy[70,73,74].

Endoscopic ultrasound-guided gastroenterostomy

Endoscopic ultrasound-guided gastroenterostomy (EUS-GE) using lumen-apposing metal stents has emerged as a safe and effective alternative method. EUS-GE can allow sustained palliation of surgical bypass while maintaining a minimally invasive endoscopic approach[75,76]. EUS-GE was first described by Binmoeller *et al*[77] in 2012, and has shown significant efficacy in palliating malignant GOO in patients who are suitable for surgical bypass[78]. In EUS-GE, a bypass is created by inserting a lumen-apposing metal stent from the stomach to the small bowel distal to the obstruction under EUS and fluoroscopic guidance.

EUS-GE can be used for palliative management of malignant GOO and can be a treatment option for benign GOO. Two recent case studies showed high technical (90%-92%) and clinical (85%-92%) success rates, with a variable percentage of adverse events (0-11.5%)[79,80]. Tyberg *et al*[79] showed there were fewer side effects (12% *vs* 41%) and similar technical success (88% *vs* 100%) with EUS-GE compared to surgical laparoscopic gastrojejunostomy. A retrospective study in 2020 by James *et al*[81] reported EUS-GE as a bridge therapy for definitive treatment of benign gastric outlet obstruction. EUS-GE was performed in 22 patients with benign GOO, and 83.3% of patients were prevented from surgery. Lumen-apposing metal stents was maintained for a mean of 8.5 mo until GOO was resolved, and the low recurrence rate of GOO (5.6%) has been reported after lumen-apposing metal stents removal. Future prospective, large-scale, randomized studies comparing surgical gastroenterostomy and EUS-GE are needed.

SURGERY

Surgery is the preferred method of treatment in patients with refractory GOO, or for whom endoscopic treatment has not been indicated. In the past, open gastrojejunostomy was widely performed, but recently laparoscopic gastrojejunostomy has become the main treatment. The laparoscopic surgical approach is more effective than open surgery for rapid postoperative recovery and is associated with a shorter hospital stay[82].

CONCLUSION

With the eradication of *H. pylori* and the use of proton pump inhibitors, the predominant causes of GOO have changed from benign to malignant diseases. Treatment of GOO depends on the underlying cause, and multiple treatment methods exist, including both endoscopic and surgical approaches. Therefore, determining the appropriate treatment for individual patients is important for treatment success and prognosis.

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ORIGINAL ARTICLE

Case Control Study

Gastrointestinal symptoms in acromegaly: A case control study

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Abstract

BACKGROUND

Acromegaly is a chronic disease caused by a pituitary somatotroph adenoma resulting in excess secretion of growth hormone, which leads to excess secretion of Insulin like growth factor 1 from the liver, causing abnormal soft tissue growth. There is increasing awareness that diseases affecting connective tissue are associated with an increase in functional gastrointestinal symptoms. Data was collected from patients with a confirmed diagnosis of acromegaly to evaluate the intensity, variety and impact of abdominal symptoms in comparison with a control group who were healthy participants recruited from the local fracture clinic.

AIM

To evaluate the frequency type and burden of abdominal symptoms in acromegaly in comparison with a control group.

METHODS

Medical documentation of patients with a diagnosis of acromegaly treated in one tertiary medical centre between 2010 and 2017 has been analysed. Data was collected from patients with confirmed acromegaly, using the Short Form Health Survey (SF36) and Rome IV Diagnostic questionnaire for Functional Gastrointestinal Disorders in Adults (R4DQ) and compared to a sex- and age-matched control group, to assess the burden of abdominal symptoms. Microsoft Excel and IBM SPSS v 25 were used for data analysis.

RESULTS

Fifty patients with acromegaly (24 male and 26 females; age range 23-64 years, mean 43) and 200 controls (96 male and 104 females; age range 18-84, mean 42.4) were recruited. 92% (46 out of 50) of patients with acromegaly reported abdominal symptoms and 78% (39 out of 50) had at least one functional gastrointestinal disorder according to the Rome IV diagnostic criteria, compared to 16% of controls (OR > 1, $P < 0.0001$). The most commonly reported symptom

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was constipation (69% acromegaly *vs* 21% of controls OR > 1, $P < 0.0001$, 95%CI: 4.4–15.8). 34 out of 50 (68%) respondents met the criteria for functional constipation according to Rome IV. Upper gastrointestinal disorders were also more prevalent in the acromegaly group. There was no statistically significant difference in the prevalence of biliary and anorectal symptoms between the two groups. Patients in acromegaly group scored lower on the mean scores of the eight parameters of SF36 Quality of Life questionnaire (mean scores 60.04 *vs* 71.23, 95%CI: -13.6829 to -8.6971, OR > 1, $P < 0.001$) as compared to the control group.

CONCLUSION

Upper and lower functional gastrointestinal tract disorders (defined by Rome IV diagnostic criteria) are significantly more prevalent in patients with acromegaly compared with healthy age and sex matched controls in our study. Functional constipation is the most commonly reported problem. Poorer quality of life may in part be attributable to the increased prevalence of abdominal symptoms.

Key words: Functional gastrointestinal disorders; Acromegaly; Constipation; Irritable bowel syndrome; Somatostatin; Pituitary

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Core tip: Irritable bowel syndrome is the commonest cause of gastrointestinal symptoms. The aetiology is thought to be multi-factorial but remains incompletely understood. Our group has previously identified that patients with connective tissue disorders have an increased incidence of functional gastrointestinal symptoms. Investigating for these symptoms in patients with acromegaly may give further insight into the pathogenesis of functional disorders and irritable bowel syndrome.

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INTRODUCTION

Acromegaly is caused by a pituitary somatotroph adenoma and characterised by excessive secretion of growth hormone (GH)[1,2]. GH stimulates the liver to produce Insulin like growth factor 1 (IGF-1). In addition to the insulin-like effects, IGF-1 can also regulate cellular DNA synthesis and is an important signalling molecule with regards to cancer cell transformation and proliferation, including mitogenesis and apoptosis inhibition[3].

A variety of complications have been reported in patients with acromegaly including cardiovascular diseases, such as hypertrophic cardiomyopathy, heart failure, hypertension, diabetes mellitus or respiratory disorders, obstructive sleep apnoea[4] as well as increased risk of benign and malignant neoplasms including colon cancer[5].

The organic gastrointestinal pathology associated with acromegaly such as increased risk of colonic cancer and an increased risk of cholelithiasis has been studied in detail[6], however the issue of overall burden of gastrointestinal symptoms, particularly the functional disorders in acromegaly and the gastrointestinal effects of its treatment have not been well studied. We have previously identified how changes in connective tissue in hypermobility (in Marfan and Ehlers Danlos) are associated with an increase in functional gastrointestinal symptoms[7]. The impact of soft tissue changes associated with over secretion of GH and gastrointestinal symptoms has not previously been studied.

Somatostatin analogues used in the treatment of acromegaly are also associated with a wide range of abdominal symptoms. Due to the higher risk of colon cancer, acromegaly patients are offered screening colonoscopy during which standard preparation for colonoscopy is often found inadequate, indicating functional and structural change[5,8,9].

Our aim was to evaluate gastro-intestinal symptoms in a cohort of acromegaly patients. We assessed the frequency, character, severity and burden of abdominal symptoms in patients with acromegaly in comparison with a control group.

MATERIALS AND METHODS

Patients

Medical documentation of patients with acromegaly treated in one medical centre (Department of Endocrinology, St George's Hospital, London) between 2010 and June 2017 have been analysed in order to find the information about their diagnosis, treatment and presence of abdominal symptoms. Treatment information including Somatostatin analogues and other medicines with significant gastrointestinal effects were obtained from patients and controls. Selected patients were then asked to fill out Rome IV Diagnostic questionnaire for Functional Gastrointestinal Disorders in Adults (R4DQ) and SF36 questionnaire and were included as cases. Results were compared with sex- and age-matched group of controls.

Controls

Participants in the control group were recruited from people who were being discharged from fracture clinic who were otherwise healthy and did not report any other medical problems. Details of treatment history including drugs affecting the gastrointestinal system were obtained.

Statistical analysis

Microsoft Excel and IBM SPSS v 25 were used to analyse the data. A case-control ratio of 4:1 was used. Fisher's exact test was used to analyse the results of R4DQ and one-tailed Independent sample t test was used to analyse the mean scores of SF36. A *P*-value under 0.05 was considered statistically significant.

Screening protocol

All patients had a confirmed diagnosis of acromegaly and were either post treatment or undergoing treatment.

Ethics

The study protocol was approved by the South West-Central Bristol Research Ethics Committee and NHS Health Research Authority United Kingdom.

RESULTS

Fifty patients with acromegaly (24 male and 26 females; age range 23-64 years, mean 43) and 200 controls (96 male and 104 females; age range 18-84, mean 42.4) were recruited in a 1:4 Case:Control ratio. The mean age at diagnosis of acromegaly was 32.44 years and on average participants had their diagnosis confirmed 11.8 years prior to this study. All patients had trans-sphenoidal surgery and 21 (42%) had pituitary radiotherapy in addition. Thirty-seven (74%) patients were using somatostatin analogues (Table 1).

Ninety-two percent (46 out of 50) of patients with acromegaly reported abdominal symptoms (abdominal pain, diarrhoea or constipation) and 78% (39 out of 50) had at least one functional gastrointestinal disorder (FGID) according to the Rome IV diagnostic criteria, compared to 16% of controls (OR > 1, $P < 0.0001$). All female patients with acromegaly reported suffering from at least some abdominal symptoms as compared to 87% of male patients with acromegaly, however there was no statistically significant gender difference observed in the frequency and intensity of symptoms. The use of medicines (antacids, histamine receptor antagonists, proton pump inhibitors, laxatives) used to alleviate gastrointestinal symptoms was also higher and statistically significant in the acromegaly group (Table 2).

A few patients with acromegaly reported multiple abdominal symptoms and qualified for more than one FGID. The most commonly reported symptom was constipation (68% acromegaly group *vs* 7.5% of controls OR > 1, $P < 0.0001$, 95%CI: 4.4-15.8) followed by abdominal pain (22% acromegaly group *vs* 9.5% of controls OR > 1, $P < 0.0001$, 95%CI: 2.5-9.3). Thirty-four out of 50 (68%) respondents met the criteria for functional constipation according to Rome IV. The prevalence of constipation increased with increasing age and was often associated with bloating. All bowel symptoms showed statistically significant prevalence in the acromegaly group. Some oesophageal and gastroduodenal conditions such as functional heartburn, functional

Table 1 Acromegaly demographic and treatment data

	Controls	Acromegaly patients
n	200	50
M:F	96:104	24:26
Age at diagnosis (mean, yr)	33	32.44
Years since diagnosis (mean, yr)	-	11.8 yr
Transsphenoidal surgery	-	50 (100%)
Pituitary radiotherapy	-	21 (42%)
Somatostatin analogue	-	37 (74%)

dysphagia and functional dyspepsia also showed statistically significant prevalence in acromegaly group. There was no statistically significant difference of prevalence of biliary and anorectal symptoms between the acromegaly group and controls (Table 3).

Acromegaly patients scored lower than controls on the mean scores of all eight parameters measured by the SF36 quality of life index. These parameters include physical functioning levels, role limitations due to physical health, role limitations due to emotional health, energy/fatigue levels, emotional wellbeing, social functioning levels, perception of pain and general health (mean scores 60.04 *vs* 71.23, 95%CI: -13.6829 to -8.6971, OR > 1, $P < 0.001$) (Table 4).

DISCUSSION

Acromegaly is a rare and unique disease associated with abnormal soft tissue growth[4] with a prevalence that is estimated at 40 per million in United Kingdom and an annual incidence rate ranging between 2 and 11 cases per million per year, with an equal distribution between genders[10].

Acromegaly is associated with gastrointestinal complications, such as constipation, higher prevalence of colorectal polyps and cancer[11] and higher prevalence of gallstones in patients treated with Somatostatin analogues[12]. Somatostatin analogues used in the management of acromegaly are also associated with a wide range of abdominal symptoms in addition to the known association with gallstones.

The higher prevalence of lower gastrointestinal symptoms in acromegaly could partly be due to slow intestinal motility. Slow intestinal and colonic transit times have been attributed to both acromegaly and its treatment with Somatostatin analogues. Disease related slow gut motility may be worsened because of treatment with Somatostatin. Resmini *et al*[13] demonstrated that patients with acromegaly have a prolonged small intestinal transit time and a prolonged colon transit time. The small bowel transit time calculated by standardized 10 g lactulose hydrogen breath test showed significantly slower oro-caecal transit in patients than in controls, without significant differences between patients treated with Somatostatin and untreated patients. These data suggest that acromegaly itself may cause motility alteration. However Thomas *et al*[14] performed radiological tests to investigate colonic transit and found an increased transit time of colon (66% longer) in patients with acromegaly compared with controls, and it was even more increased during octreotide treatment. This may predispose to small intestinal bacterial overgrowth, which in turn can cause symptoms[13]. Autonomic intestinal impairment due to vagal hypertonia, similar to that demonstrated previously in the cardiovascular system, has been proposed as a pathogenic mechanism[15]. Another proposed pathogenic mechanism could be related to hormonal imbalance which can be influenced by the complex interaction between GH and ghrelin, as shown by Arosio *et al*[16].These gut motility disturbances in acromegaly increase circulating levels of IGF-1, which is a known mitogen[17] that may stimulate the proliferation of intestinal epithelial cells by autocrine and paracrine actions[11].

Our study has also showed a higher prevalence of upper gastrointestinal symptoms (functional heartburn, functional dysphagia and functional dyspepsia) in the acromegaly group. Sisman *et al*[18] showed that the prevalence of gastritis, duodenitis, peptic ulcers or intestinal metaplasia were higher in patients with acromegaly than in healthy subjects; while the prevalence of hiatal hernia was lower. Ilhan *et al*[19] demonstrated oesophageal dysmotility manifesting as profound reduction in the amplitude and duration of lower oesophageal sphincter relaxation even in acromegaly patients without any significant gastrointestinal symptoms. George *et al*[20] described a rare case of megaduodenum without any distal obstruction in a patient

Table 2 Acromegaly abdominal symptoms and medicine use

		Controls (*n* = 200)	Acromegaly patients (*n* = 50)	*P* value (Fisher's exact test)
Gastrointestinal symptoms		32 (16%)	46 (92%)	
	Abdominal pain	19 (9.5%)	11(22%)	0.4905
	Diarrhoea	8 (4%)	4 (8%)	0.2652
	Constipation	15 (7.5%)	34 (68%)	< 0.00001[b]
Medicines				
	Regular use of anti-secretory (PPI/H2RA)/antacids	11 (6.5%)	31 (62%)	< 0.00001[b]
	Regular use of laxatives	6 (3%)	46 (92%)	< 0.00001[b]
	Regular use of opioid analgesics	11 (6.5%)	2 (4%)	Not statistically significant
	Somatostatin analogues	-	37 (74%)	

[b]*P* < 0.01, statistically significant. PPI: Proton pump inhibitor; H2RA: Histamine H2 receptor antagonists.

with acromegaly. Somatostatin analogues inhibitory effect on gut motility, particularly antral contractility may also produce or worsen symptoms in patients with delayed gastric emptying[21].

Despite our study not showing any statistically significant difference in the prevalence of biliary symptoms in the two groups, other studies have shown an increased risk of gall stones in acromegaly[22-24]. The increased risk of faecal anaerobic bacteria overgrowth associated with slow gut transit times, with the additional increased risk of impairment of bile acid metabolism may be responsible in part for gallstone development[14]. Ultrasound studies have found increased gall bladder volume in both fasting and postprandial states in acromegaly. These are associated with profound suppression of released cholecystokinin, which is associated with Somatostatin administration. The incomplete gall bladder emptying may be the reason for higher incidence of gall bladder calculi in patients with acromegaly treated with Somatostatin[12].

This study is the first study in our knowledge that looks into the prevalence of various abdominal symptoms in acromegaly. The burden of non-organic gastrointestinal symptoms, particularly the functional disorders in acromegaly and the gastrointestinal effects of its treatment have not been well studied. This study, along with our previous studies on Marfan and Ehlers Danlos[7] gives insight into the possible link between connective tissue abnormalities and irritable bowel syndrome (IBS). IBS is the commonest final diagnosis in patients presenting with gastrointestinal symptoms. The aetiology is multifactorial. This study gives further evidence to the suggestion that connective tissue abnormalities may be the underlying pathology in some individuals with IBS.

The strengths of this study are that for a very rare condition this is a large patient cohort being looked after in one tertiary hospital, with a large control group for comparison. A weakness of this study is that cases were not clinically reviewed to ensure that symptoms had been fully investigated to ensure that the symptom complex was truly functional. Also, it was not possible to analyse for disease duration prior to diagnosis, treatment administered historically, on-going treatment and time since diagnosis were not possible.

The presence of FGIDs affecting both upper and lower gastrointestinal tract in patients with acromegaly is substantially higher than the controls in our study. The lower mean scores on quality of life indicators in the acromegaly group reflect the overall burden of disease on quality of life. The high prevalence of abdominal symptoms may in part explain this. This is likely to be multifactorial and factors such as delayed small intestinal and colonic transit times, treatment with Somatostatin analogues, increased bowel length may all play a part in this. A larger follow up international multi-centre study on the presence of abdominal symptoms in acromegaly and future clinical research focussing on the association of abdominal symptoms with connective tissue abnormalities may further help our understanding of IBS and other FGIDs.

Table 3 Gastrointestinal symptoms in acromegaly patients compared with controls

		Controls, *n* = 200 (%)	Acromegaly patients, *n* = 50 (%)	*P* value (Fisher's exact test)
Oesophageal disorders				
	Functional chest pain	2 (1%)	3 (6%)	0.0561
	Functional heartburn	22 (11%)	15 (30%)	0.0016[b]
	Globus	2 (1%)	2 (4%)	0.1796
	Functional dysphagia	4 (2%)	6 (12%)	0.0053[b]
Gastroduodenal disorders				
	Functional dyspepsia	14 (7%)	12 (24%)	0.0013[b]
	Belching disorders	8 (4%)	5 (10%)	0.1441
	Nausea and vomiting disorders	2 (1%)	2 (4%)	0.1796
	Rumination syndrome	2 (1%)	1 (2%)	0.4895
Bowel disorders				
	Irritable bowel syndrome	12 (6%)	10 (20%)	0.0041[b]
	Functional constipation	22 (11%)	34 (68%)	0.0006[b]
	Functional diarrhoea	10 (5%)	9 (18%)	0.0048[b]
	Functional abdominal bloating/distension	12 (6%)	11 (22%)	0.0015[b]
	Unspecified functional bowel disorder	44 (22%)	25 (50%)	0.0002[b]
Centrally mediated Abdominal pain syndrome		6 (3%)	4 (8%)	0.1165
Functional Biliary pain		1 (0.5%)	3 (6%)	0.0261[a]
Anorectal disorders				
	Faecal incontinence	1 (0.5%)	2 (4%)	0.1028
	Functional anorectal pain	2 (1%)	4 (8%)	0.0157[a]

[a]$P < 0.05$,
[b]$P < 0.01$, statistically significant.

Table 4 Quality of life scores in acromegaly patients

	Controls (mean scores)	Acromegaly (mean scores)	Independent sample *t* test
Physical functioning	100.0	80.0	0.80516
Role limitations due to physical health	100.0	50.0	0.001053[b]
Role limitations due to emotional problems	100.0	100.0	1
Level of energy/fatigue	95.0	45.0	0.001053[b]
Emotional wellbeing	100.0	68.0	0.002175[b]
Social functioning	87.5	50.0	< 0.000076[b]
Pain	77.5	67.5	0.1732
General health	100	30.0	< 0.0001[b]

[b]$P < 0.01$, statistically significant.

ARTICLE HIGHLIGHTS

Research background

Acromegaly results from an excess of growth hormone, which leads to excess secretion of Insulin like growth factor 1 from the liver, causing abnormal soft tissue growth. This is associated with an increase in a number of organic diseases. There is increasing awareness that diseases affecting connective tissue are associated with an increase in functional gastrointestinal symptoms. We are not aware of any other study that has looked into the burden of abdominal symptoms in acromegaly and the impact they have on patient's quality of life as a result of this.

Research motivation

This study is part of a larger study that is assessing the role and significance of connective tissue involvement in abdominal symptoms. We have previously described an increase in functional gastrointestinal symptoms in other diseases affecting connective tissue (Marfan and Ehlers Danlos). In this study we evaluate the frequency of abdominal symptoms in patients with acromegaly.

Research objectives

The main objective of this study was to evaluate if patients with acromegaly had more frequent and intense abdominal symptoms, as described by the Rome criteria, than controls and thus, as a result of this had poorer quality of life. Furthermore, other factors such as use of Somatostatin analogues, which in itself can cause abdominal symptoms, had to be taken into account. The next step in our research would be to carry out objective gastrointestinal physiological studies in larger groups of patients and controls to see if the presence of symptoms reflects actual physiological variations across different groups.

Research methods

Patients with acromegaly were identified from a clinical database at one tertiary medical centre (Department of Endocrinology, St George's Hospital, London). Identified patients were then asked to fill out previously validated questionnaires and results were compared with sex- and age-matched group of controls (recruited from fracture clinic who were otherwise healthy and did not report any other medical problems).

Research results

The results of this study showed that the presence of abdominal symptoms in acromegaly is significantly higher than controls. The results also show that the presence of these symptoms has an overall detrimental effect on quality of life or well being of the patient. This study also supports the increasing awareness in the scientific world regarding the association of connective tissue abnormalities and gastrointestinal or abdominal symptoms. It is yet to be seen if gastrointestinal physiological studies in these patients will be reflective of these results.

Research conclusions

The presence of abdominal symptoms is significantly higher in patients with acromegaly compared to controls and this may have a significant impact on their quality of life. Connective tissue abnormalities are associated with abdominal symptoms as has been shown by this study and other studies and may be one of the underlying reasons behind functional gastrointestinal disorders (FGIDs). Other studies have shown similar results in Ehlers-Danlos and Marfan syndromes and abnormalities of connective tissue such as elastin may be common to these disease processes. This needs to be studied further to see if minor variations in gut connective tissue the cause of FGIDs could be.

Research perspectives

Future research in this area will have to be pursued in an international and multicentre study as it is difficult for one centre to recruit a large number of patients in rare diseases. Gastrointestinal physiological studies would help to see if the variance in symptoms is reflected in physiological variance.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Validation of American Joint Committee on Cancer 8th edition of TNM staging in resected distal pancreatic cancer

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Abstract

statement: This study was reviewed and approved by the Institutional Review Board of University of Florida.

Informed consent statement: Patients were not required to give informed consent to the study because our study was done retrospectively and data for study obtained after each patient agreed to treatment. Informed consent waiver was approved by Institutional review board of University of Florida.

Conflict-of-interest statement: The authors declare no competing interests in this study.

Data sharing statement: No additional data are available.

STROBE statement: The manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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BACKGROUND
In order to improve risk stratification and clinical management of the pancreatic ductal adenocarcinoma (PDAC), the American Joint Committee on Cancer (AJCC) has published its eighth edition staging manual. Some major changes have been introduced in the new staging system for both T and N categories. Given the rarity of resectable disease, distal pancreatic cancer is likely underrepresented in the published clinical studies, and how the impact of the staging system actually reflects on to clinical outcomes remain unclear.

AIM
To validate the AJCC 8th edition of TNM staging in distal PDAC.

METHODS
A retrospective cohort study was performed in seven academic medical centers in the United States. Clinicopathological prognostic factors associated with progression-free survival (PFS) and overall survival (OS) were evaluated through univariate and multivariate analyses.

RESULTS
Overall, 454 patients were enrolled in the study, and were divided into 2 subgroups: Invasive intraductal papillary mucinous neoplasms (IPMN) (115 cases) and non-IPMN associated adenocarcinoma (339 cases). Compared to invasive IPMN, non-IPMN associated adenocarcinomas are more common in relatively younger patients, have larger tumor size, are more likely to have positive lymph nodes, and are associated with a higher tumor (T) stage and nodal (N) stage, lymphovascular invasion, perineural invasion, tumor recurrence, and a worse PFS and OS. The cohort was predominantly categorized as stage 3 per AJCC 7th edition staging manual, and it's more evenly distributed based on 8th edition staging manual. T and N staging of both 7th and 8th edition sufficiently stratify PFS and OS in the entire cohort, although dividing into N1 and N2 according to the 8th edition does not show additional stratification. For PDAC arising in IPMN, T staging of the 7th edition and N1/N2 staging of the 8th edition appear to further stratify PFS and OS. For PDAC without an IPMN component, T staging from both versions fails to stratify PFS and OS.

CONCLUSION
The AJCC 8th edition TNM staging system provides even distribution for the T staging, however, it does not provide better risk stratification than previous staging system for distal pancreatic cancer.

Key words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Prognosis; Intraductal papillary mucinous neoplasms; Survival; American Joint Committee on Cancer

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Core tip: The American Joint Committee on Cancer 8th edition TNM staging system provides even distribution for the T staging, however, it does not provide better risk stratification than previous staging system for distal pancreatic cancer. This study also demonstrates the significant difference of clinical outcome and risk stratification between invasive intraductal papillary mucinous neoplasms and non-intraductal papillary mucinous neoplasms associated pancreatic ductal adenocarcinoma.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers. In the United States, PDAC is the fourth leading cause of cancer related deaths causing approximately 7% of all cancer mortalities, and has a dismal 5-year survival rate of less than 10%[1,2]. A strong contributor to the low overall survival (OS) rate is a lack of early diagnostic symptoms, which results in PDAC frequently presenting at a late stage with locally advanced or metastatic disease[3].

In order to improve risk stratification, staging, and clinical management of PDAC, the American Joint Committee on Cancer (AJCC) published its eighth edition staging manual for pancreatic cancer in October 2016[4]. Some major changes have been introduced in the new staging system for both T and N categories. Of note, tumor size has become the only factor for the T staging except for pT4. pT1 is further subclassified into T1a (tumor 0.5 cm or less), T1b (tumor greater than 0.5 cm and less than 1 cm), and T1c (tumor greater than 1 cm but no more than 2 cm). The most important change was to replace the requirement of extra-pancreatic extension with tumor size (tumor greater than 4 cm) for a pT3 tumor. For the N category, N1 (regional lymph node metastasis) is subclassified into N1 (metastasis in 1-3 nodes) and N2 (metastasis in 4 or more nodes) in 8th edition staging system[5]. Given the rarity of resectable disease, distal pancreatic cancer is likely underrepresented in the published clinical studies, and how the impact of the staging system actually reflects on to clinical outcomes remain unclear.

Intraductal papillary mucinous neoplasm (IPMN) is a pancreatic neoplasm with different epithelial types and histopathological grades[6,7] that has the potential to progress into invasive carcinoma. The invasive IPMN and non-IPMN associated PDAC are likely derived through different molecular pathways[8,9], and reports have suggested improved survival of patients with invasive IMPN as compared to patients with non-IPMN associated PDAC[10,11]. However, whether invasive IPMN and non-IPMN PDAC share similar prognostic factors still remains unclear. So far, there is no specific staging system for invasive IPMN. Conventionally, it is staged using the AJCC system primarily developed and validated for non-IPMN associated PDAC. The size of the invasive component in invasive IPMN is subjective due to variability in sampling and measurement techniques and may not be a reliable parameter for staging.

Until now, the majority of validation studies for AJCC 8th edition were performed in patients with solely or predominantly PDAC in the head of pancreas[5,12-16]. The current study was designed to validate the major T and N staging changes by the AJCC 8th edition of TNM staging manual in a cohort of patients with resected distal pancreatic carcinoma, as well as in a sub-cohort of patients with invasive IPMN or non-IPMN associated PDAC. Univariate and multivariable survival analyses were performed to identify prognostic factors in a multi-centered large-scale study.

MATERIALS AND METHODS

Patient characteristics

After approval from Institutional Review Boards from individual institutions, a retrospective cohort study was performed with cases collected from seven academic medical centers in the United States (University of Florida, Indiana University, Dartmouth-Hitchcock Medical Center, Penn State Health Hershey Medical Center, Thomas Jefferson University Hospital, University of Rochester Medical Center, and Yale University).

Clinicopathological data of resected distal PDAC cases were retrieved from year 2005 to 2018. Other tumor subtypes such as non-invasive IPMN, non-invasive mucinous cystic neoplasm, neuroendocrine neoplasm, and acinar cell carcinoma were excluded. Those cases which underwent preoperative neoadjuvant therapy were excluded from this study.

Histopathology review

Histopathological data were collected through reviewing pathology reports and glass slides. Oncological information was collected including tumor size, type, differentiation, margin status, splenic vasculature involvement, and lymph node status. All cases were re-staged based on the AJCC 7th and 8th edition, respectively. Splenic vein involvement was defined as tumor invading through the venous wall. Splenic artery involvement was defined as tumor invading into or through the arterial wall.

Clinical information review

Clinical information including patient's age, gender, radiographic studies, recurrence, metastasis, and survival status was collected through reviewing the medical records from the time of resection until September 2018. The progression free survival (PFS) is defined as the interval between the date of surgery and the date of initial recurrence and/or metastasis of tumor or death. The OS is defined as the interval between the date of surgery and the date of death.

Statistical analysis

Continuous variables were summarized as median and interquartile range (IQR) and compared with Wilcoxon rank sum test. Categorical variables were summarized as counts and percentages and compared with Fisher's exact test. PFS and OS were evaluated using Kaplan-Meier curves, and analyzed using log-rank analysis. Cox proportional hazards regression models were used in univariate and multivariable survival analyses to identify factors associated with PFS and OS. The proportionality assumption was assessed graphically using log (-log) plots and quantitatively using the Z statistic. All tests were two-sided and performed in R (version 3.5.3). A P value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The study enrolled 454 patients with resected distal pancreatic cancer. The age of the patients ranged from 27 to 91 years, with a mean age of 67.1 years, 53.5% of the patients were female (n = 243), and 46.5% were male (n = 211). Pathologic demographics and major pathologic features are summarized in Table 1.

Stratification of patient survival in entire cohort

The majority of patients were categorized as stage pT3 per AJCC 7th edition staging manual, with 32 patients (7.0%), 50 patients (11.0%), and 351 patients (77.3%) being classified as pT1, pT2, and pT3, respectively. When categorizing based upon the AJCC 8th edition staging manual, the distribution was relatively more even, with 76 patients (16.7%), 202 patients (44.5%), and 166 patients (36.6%) classified as pT1, pT2, and pT3, respectively. T staging of both 7th and 8th systems sufficiently stratified PFS and OS in the entire cohort (Figure 1A, B, E and F).

Among the 237 patients with positive lymph nodes (pN1 per 7th edition), 180 patients (75.9%) were classified as pN1, and 57 patients (24.1%) were classified as pN2 based on AJCC 8th edition. N staging of both 7th and 8th systems sufficiently stratified PFS and OS in the entire cohort, although dividing into N1 and N2 according to the 8th edition did not show additional stratification (Figure 1C, D, G and H).

Invasive IPMN vs non-IPMN-associated adenocarcinoma

The diagnostic group (IPMN *vs* non-IPMN) is an important prognostic factor in resected distal pancreatic cancer. In the cohort, 115 patients (25.3%) and 339 patients (74.7%) were diagnosed as invasive IPMN and non-IPMN associated PDAC, respectively. There was no gender difference between the 2 groups, with a slight female predominance in both groups (53.0% female in IPMN group vs 53.7% in non-IPMN group). Compare to the invasive IPMN group, non-IPMN associated PDAC are more commonly seen in relatively younger patients (median age 66.7 years in non-IPMN group *vs* 70.0 years in IPMN group; P = 0.003), and have larger tumor size [median tumor size 3.6 cm (IQR: 2.5-5.2) in non-IPMN group *vs* median tumor size 3.0 cm (IQR: 2.0-4.5) in IPMN group; P = 0.012]. Non-IPMN associated PDAC are also associated with higher tumor (T) stage and nodal (N) stage, lymphovascular invasion (LVI), perineural invasion, tumor recurrence, as well as worse PFS and OS (Table 1).

Prognostic factors and stratification of patient survival with invasive IPMN

A total of 115 patients with invasive IPMN were included in the study. Sixty-one patients (54.0%) were female and fifty-four (47.0%) were male, with the median age of 70.0 years (Table 1).

In univariable analysis for invasive IPMN, tumor size, poor differentiation, pT3 stage (8th edition staging manual), positive lymph node, pN2 stage (8th edition staging manual), and pathologic splenic vein invasion were significantly associated with both poor PFS and poor OS. LVI, splenic artery invasion, and splenic parenchyma invasion were only associated with PFS. Positive resection margin and perineural invasion were only associated with OS. In multivariable analysis, only pT3 (8th edition staging manual) remained as independent prognosticator for OS (Table 2).

Table 1 Clinical and pathological characteristics of pancreatic ductal adenocarcinoma patients

Feature	Level	Total cohort	Subcohort		
			Invasive IPMN	Non-IPMN associated PDAC	*P* value
		Median (IQR)			
Age (in yr)		67.6 (60.0, 74.8)	70.0 (64.1, 75.5)	66.7 (59.4, 74.0)	0.003
Tumor size (in cm)		3.5 (2.5, 5.0)	3.0 (2.0, 4.5)	3.6 (2.5, 5.2)	0.012
		Median (95%CI)			
PFS (in mo)		21.0 (17.0, 27.0)	NR (47.0, NR)	17.0 (13.0, 22.0)	< 0.001
OS (in mo)		21.0 (19.0, 24.0)	60.0 (28.0, 69.0)	19.0 (18.0, 22.0)	< 0.001
		n (%)			
Gender (male/female)	Female	243 (53.5)	61 (53.0)	182 (53.7)	0.991
	Male	211 (46.5)	54 (47.0)	157 (46.3)	
Tumor differentiation	Well	41 (9.0)	15 (13.4)	26 (7.9)	0.103
	Moderate	271 (59.7)	71 (63.4)	200 (61.0)	
	Poor	128 (28.2)	26 (23.2)	102 (31.1)	
AJCC 8th ed staging system	T1	76 (16.7)	32 (27.8)	44 (13.0)	0.003
	T2	202 (44.5)	46 (40.0)	156 (46.2)	
	T3	166 (36.6)	36 (31.3)	130 (38.5)	
AJCC 8th ed staging system	N0	216 (47.6)	69 (60.0)	147 (43.4)	0.004
	N1	181 (39.9)	31 (27.0)	150 (44.2)	
	N2	57 (12.6)	15 (13.0)	42 (12.4)	
Lymphovascular invasion	Positive	219 (48.2)	44 (38.3)	175 (51.6)	0.011
	Negative	219 (48.2)	69 (60.0)	150 (44.2)	
	Indeterminant	16 (3.5)	2 (1.7)	14 (4.1)	
Perineural invasion	Positive	366 (80.6)	80 (69.6)	286 (84.4)	0.001
	Negative	82 (18.1)	34 (29.6)	48 (14.2)	
	Indeterminant	6 (1.3)	1 (0.9)	5 (1.5)	
Recurrence/metastasis	Present	218 (48.0)	43 (37.4)	175 (51.6)	0.015
	Absent	236 (52.0)	72 (62.6)	164 (48.4)	
Splenic parenchymal invasion	Present	25 (5.5)	2 (1.7)	23 (6.9)	0.067
	Absent	425 (93.6)	113 (98.3)	312 (93.1)	

AJCC: American Joint Committee on Cancer; IPMN: Intraductal papillary mucosal neoplasm; PDAC: Pancreatic ductal adenocarcinoma; PFS: Progression-free survival; OS: Overall survival; IQR: Interquartile range; NR: Not reached.

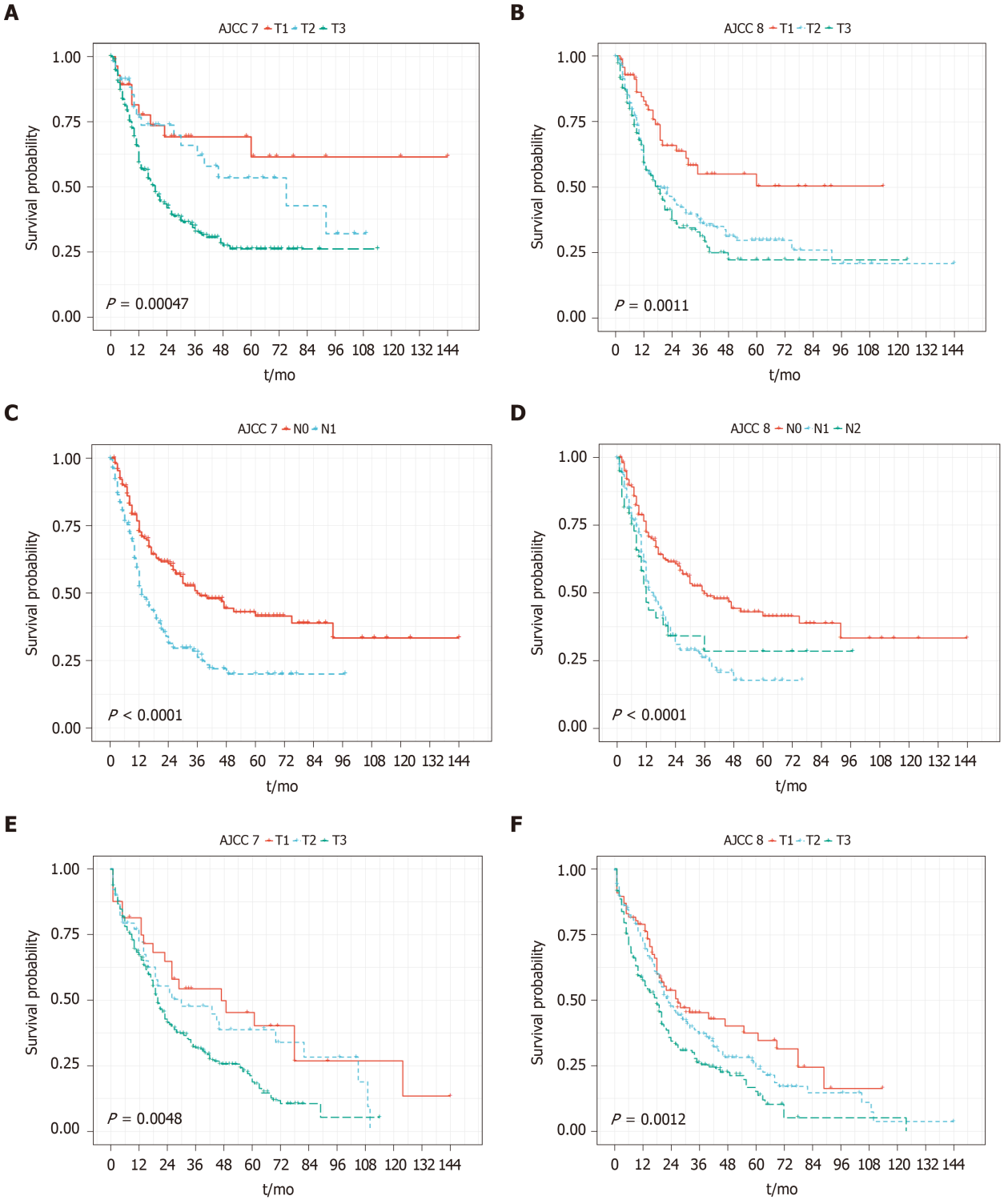
Per AJCC 7th edition staging manual, 13 patients (11.3%), 21 patients (18.3%), and 77 patients (67.0%) were classified as pT1, pT2, and pT3, respectively. Per AJCC 8th edition, 32 patients (27.8%), 46 patients (40.0%), and 36 patients (31.3%) were classified as pT1, pT2 and pT3, respectively. Among 46 patients with lymph node metastasis (pN1 per AJCC 7th edition), 31 patients and 15 patients were further diagnosed with pN1 and pN2 tumor according to AJCC 8th edition. T staging of the 7th system appeared to further stratify PFS and OS, however N1/N2 staging of the 8th edition appeared to further stratify PFS and OS (Figure 2).

Stratification of patient survival with non-IPMN associated PADC

A total of 339 patients with non-IPMN associated PDAC were included in the study. One hundred eighty-two patients (53.7%) were female and one hundred fifty-seven (46.3%) were male, with the median age of 66.7 years (Table 1).

In univariable analysis for non-IPMN associated PADC, nodal metastasis, pN1 stage (8th edition), and LVI were significantly associated with both PFS and OS. Tumor size, pathologic or radiographic evidence of splenic vein invasion, and adjuvant chemotherapy were all significantly associated with OS. In multivariable analysis, however, only pN1 (8th edition staging manual) remained as an independent prognosticator for both PFS and OS, while adjuvant chemotherapy remained as independent prognosticator for OS only (Table 3).

Per AJCC 7th edition staging manual, 19 patients (5.6%), 29 patients (8.6%), and 274


A
AJCC 7 T1 T2 T3
Survival probability
1.00
0.75
0.50
0.25
0.00
P = 0.00047
0 12 24 36 48 60 72 84 96 108 120 132 144
t/mo
B
AJCC 8 T1 T2 T3
Survival probability
P = 0.0011
t/mo
C
AJCC 7 N0 N1
Survival probability
P < 0.0001
t/mo
D
AJCC 8 N0 N1 N2
Survival probability
P < 0.0001
t/mo
E
AJCC 7 T1 T2 T3
Survival probability
P = 0.0048
t/mo
F
AJCC 8 T1 T2 T3
Survival probability
P = 0.0012
t/mo

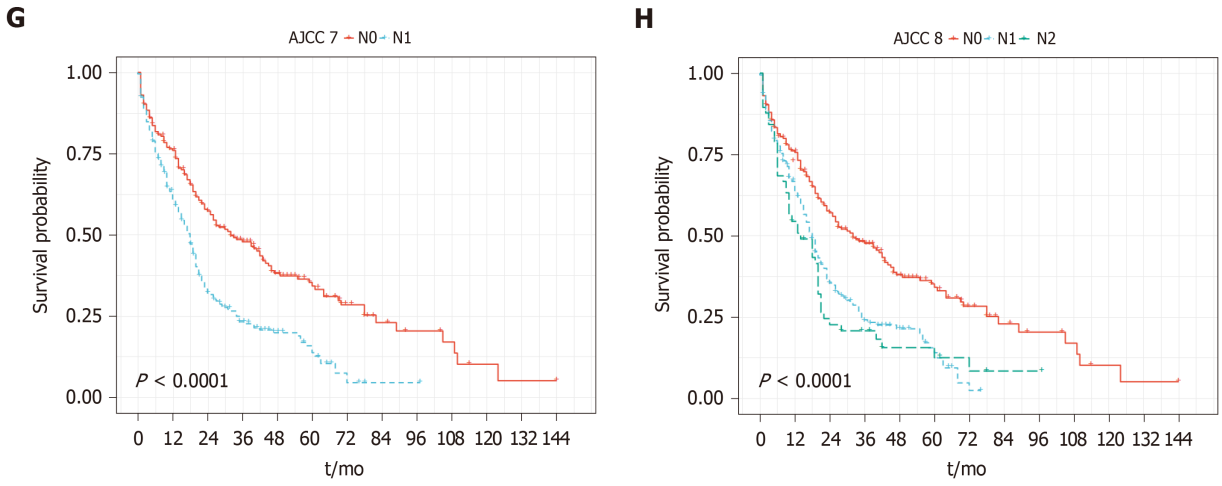


Figure 1 Kaplan-Meier curves for survival of the resected distal pancreatic cancer. A: PFS stratified according to AJCC 7th edition T staging; B: PFS stratified according to the AJCC 8th edition T staging; C: PFS stratified according to AJCC 7th edition N staging; D: PFS stratified according to the AJCC 8th edition N staging; E: OS stratified according to AJCC 7th edition T staging; F: OS stratified according to the AJCC 8th edition T staging; G: OS stratified according to AJCC 7th edition N staging; H: OS stratified according to the AJCC 8th edition N staging. PFS: Progression-free survival; AJCC: American Joint Committee on Cancer; OS: Overall survival.

patients (80.8%) were classified as pT1, pT2, and pT3, respectively. Per AJCC 8th edition staging manual, 44 patients (13.0%), 149 patients (44.0%), and 130 patients (38.3%) were classified as pT1, pT2 and pT3, respectively. Among 191 patients with lymph node metastasis (pN1 per AJCC 7th edition staging manual), 149 patients and 42 patients were further sub classified into pN1 and pN2 tumor according to AJCC 8th edition. For PDAC without IPMN component, T staging from both staging systems failed to stratify PFS and OS. N staging of both staging systems could stratify PFS and OS, although dividing N category into pN1 and pN2 as in the 8th edition staging manual did not add further value to this group (Figure 3).

DISCUSSION

In this study, we evaluated the prognostic relevance of the 8th edition AJCC TNM staging manual in a large United States cohort of distal pancreatic cancer. The new staging system recognizes tumor size as one of the most important prognostic factors for tumor staging. In practice, the vast majority of PDAC cases had spread into the peripancreatic soft tissue and were staged as pT3 even after the introduction of the 5th edition of the AJCC staging manual in 1997. By removing the diagnostic criteria of extra pancreatic extension for pT3, patients with PDAC are distributed more evenly using the new AJCC 8th staging system.

Even with relatively strict selection criteria, wide heterogeneity of diagnostic groups was still inevitable within our study subjects. To simply this issue, we classified the cases as invasive IPMN and non-IPMN-associated adenocarcinoma. Consistent with previous reports[10,11], invasive IPMN is associated with lower T stage and N stage, as well as better survival in our study. The study revealed multiple independent prognostic factors for patients with distal pancreatic cancer, such as poor differentiation, tumor size, pT3 stage (8th edition staging manual), positive lymph node, pN2 stage (8th edition staging manual), LVI, splenic artery or vein invasion, positive resection margin, and splenic parenchymal invasion. Interestingly, very limited overlap was observed among patients with invasive IPMN and non-IPMN associated PDAC. For invasive IPMN patients, pT3 is the only significant prognostic factor for OS upon multivariable analysis. On the other hand, lymph node metastasis (pN1 stage per 8th edition) appears to be the only independent prognosticator for both PFS and OS in non-IPMN-assoiciated adenocarcinoma. The underlying mechanism account for these differences as well as their distinct clinical behavior and survival is not clear, but the molecular pathways underlying tumorigenesis for each entity might provide clues in the future.

Consistent with a recent study[16], our study demonstrates that the AJCC 7th edition T staging better stratifies survival in patients with invasive IPMN. Tumor size alone is not an independent prognostic factor based on multivariable analysis[16]. It's interesting to identify pT3 (8th edition) as the only significant risk factor for survival in

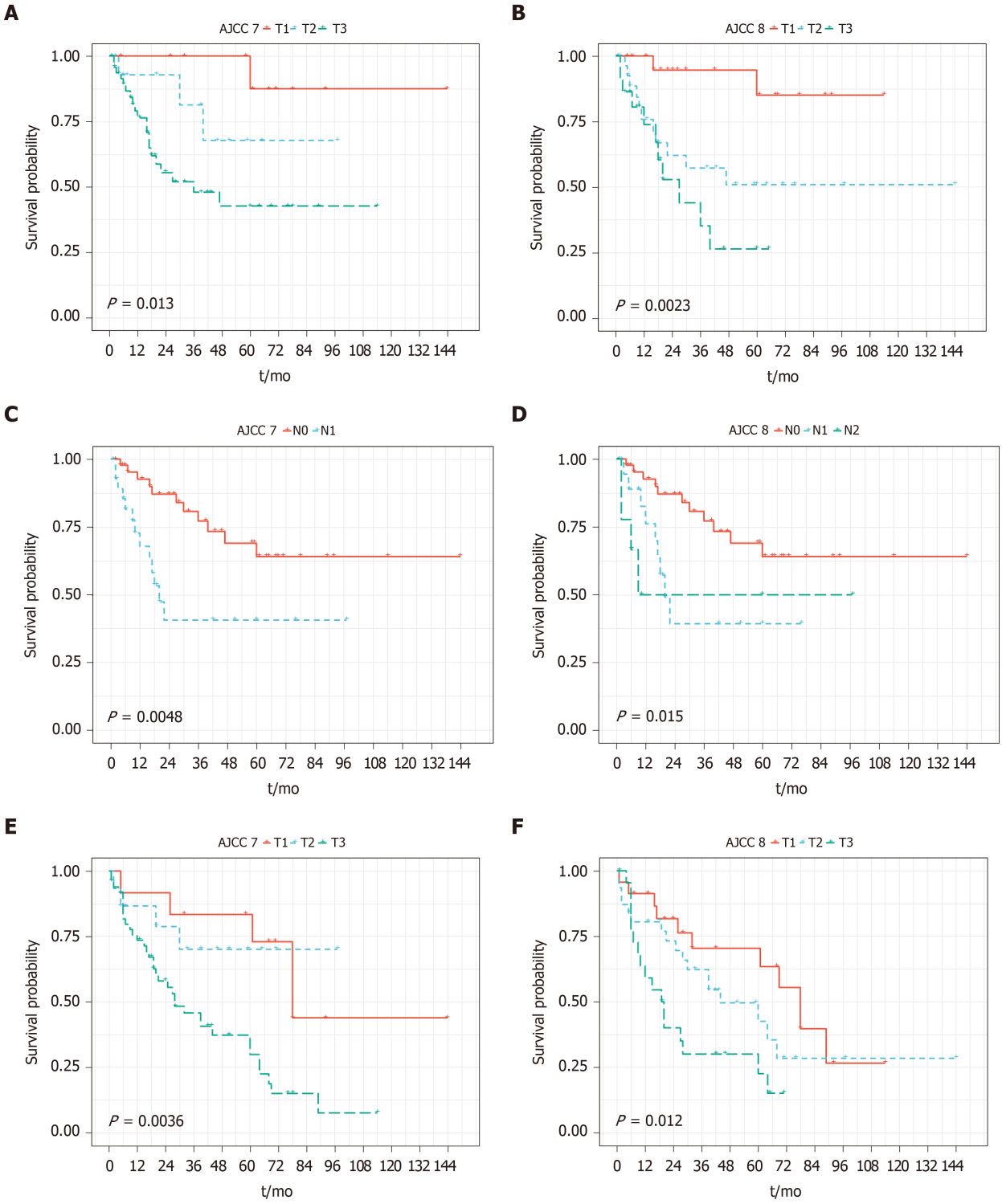
Table 2 Univariate and multivariable analysis for progression-free survival and overall survival in invasive intraductal papillary mucinous neoplasm

Feature	Level	PFS		OS	
		HR (95%CI), *P* value			
Gender		1.67 (0.91-3.05), *P* = 0.10	-	1.33 (0.85-2.08), *P* = 0.22	-
Age		0.99 (0.96-1.02), *P* = 0.52	-	1.01 (0.98-1.03), *P* = 0.54	-
Tumor size		1.18 (1.05-1.32), *P* = 0.01	-	1.10 (1.00-1.21), *P* = 0.04	-
Tumor differentiation	Well	-	-	-	-
	Moderate	2.71 (0.82-8.91), *P* = 0.10	12290265.82 (0.00-Inf), *P* = 1.00	1.04 (0.53-2.02), *P* = 0.92	1.62 (0.34-7.66), *P* = 0.55
	Poor	3.88 (1.06-14.27), *P* = 0.04	2274054.50 (0.00-Inf), *P* = 1.00	2.31 (1.10-4.86), *P* = 0.03	1.78 (0.15-20.67), *P* = 0.64
Positive lymph node		1.19 (1.05-1.35), *P* = 0.01	-	1.19 (1.07-1.31), *P* < 0.01	-
T stage (AJCC 8th edition staging manual)	T1	-	-	-	-
	T2	2.69 (1.14-6.37), *P* = 0.02	0.81 (0.07-9.31), *P* = 0.87	1.65 (0.92-2.97), *P* = 0.09	0.89 (0.09-8.48), *P* = 0.92
	T3	3.48 (1.42-8.51), *P* = 0.01	5.29 (0.44-62.97), *P* = 0.19	2.28 (1.23-4.24), *P* = 0.01	26.22 (2.13-322.77), *P* = 0.01
N stage (AJCC 8th edition staging manual)	N0	-	-	-	-
	N1	2.03 (1.03-4.02), *P* = 0.04	13.78 (1.15-165.63), *P* = 0.04	1.58 (0.93-2.67), *P* = 0.09	1.17 (0.24-5.70), *P* = 0.85
	N2	3.65 (1.60-8.34), *P* < 0.01	29.12 (0.87-979.78), *P* = 0.06	2.52 (1.36-4.65), *P* < 0.01	1.63 (0.10-27.11), *P* = 0.73
Lymphovascular invasion		2.12 (1.16-3.90), *P* = 0.02	0.31 (0.04-2.41), *P* = 0.26	1.43 (0.90-2.28), *P* = 0.13	-
Perineural invasion		1.99 (0.98-4.04), *P* = 0.06	-	2.45 (1.39-4.33), *P* < 0.01	2.67 (0.54-13.22), *P* = 0.23
Splenic artery invasion		5.30 (1.41-19.94), *P* = 0.01	2.44 (0.10-62.16), *P* = 0.59	1.70 (0.59-4.88), *P* = 0.32	-
Splenic vein invasion		18.69 (4.57-76.48), *P* < 0.01	5.28 (0.22-129.25), *P* = 0.31	2.66 (1.08-6.55), *P* = 0.03	5.62 (0.61-51.99), *P* = 0.13
Positive margin		1.25 (0.30-5.19), *P* = 0.76	-	3.14 (1.49-6.62), *P* < 0.01	0.00 (0.00-Inf), *P* = 1.00
Splenic parenchyma invasion		14.93 (1.83-121.81), *P* = 0.01	1.00 (1.00-1.00), *P* = NaN	1.23 (0.17-8.87), *P* = 0.84	-
Adjuvant radiation therapy		0.97 (0.52-1.81), *P* = 0.92	-	0.81 (0.48-1.37), *P* = 0.43	-
Adjuvant chemotherapy		2.50 (0.89-7.00), *P* = 0.08	-	0.69 (0.39-1.22), *P* = 0.20	-
Radiographic splenic artery invasion		2.12 (0.85-5.29), *P* = 0.11	-	1.45 (0.59-3.56), *P* = 0.41	-
Radiographic splenic vein invasion		2.27 (1.04-4.94), *P* = 0.04	2.88 (0.33-24.96), *P* = 0.34	1.46 (0.68-3.13), *P* = 0.33	-

AJCC: American Joint Committee on Cancer; PFS: Progression-free survival; OS: overall survival.

our study. One possible explanation is that majority of the pT3 tumor (> 4 cm per 8th edition staging manual) extend into peripancreatic soft tissue, and would also be staged as pT3 per the 7th edition staging manual[17]. In contrast to the T staging, N1/N2 staging of the 8th edition staging manual appears to further stratify PFS and OS in invasive IPMN.

An unexpected finding in this study is that T staging from both staging systems failed to stratify PFS and OS in resected distal non-IPMN associated PDAC. Multiple studies have demonstrated the clinical relevance, reproducibility, and risk stratification of the AJCC 8th edition staging manual[5,12-15]. However, due to the relative rarity, resected distal PDAC is likely underrepresented among those published studies. It should be noted that the stage-independent OS in distal pancreatic cancer is much worse as compared to its counterpart in the pancreatic head, and tumor location itself has been considered as a prognostic factor for survival in pancreatic cancer patients[18,19]. Delay in diagnosis is likely the major reason for its poor prognosis, although other facts may also play a role. For example, distal pancreatic cancer patients are significantly older at the time of diagnosis, and less dissectible lymph nodes are present at the distal portion of the pancreas[19]. Unlike invasive IPMN, lymph node metastasis is the most important prognostic factor for survival in non-IPMN associated PDAC. N staging of both 7th and 8th edition could stratify PFS and


A
AJCC 7 T1 T2 T3
Survival probability
1.00
0.75
0.50
0.25
0.00
P = 0.013
0 12 24 36 48 60 72 84 96 108 120 132 144
t/mo
B
AJCC 8 T1 T2 T3
Survival probability
P = 0.0023
t/mo
C
AJCC 7 N0 N1
Survival probability
P = 0.0048
t/mo
D
AJCC 8 N0 N1 N2
Survival probability
P = 0.015
t/mo
E
AJCC 7 T1 T2 T3
Survival probability
P = 0.0036
t/mo
F
AJCC 8 T1 T2 T3
Survival probability
P = 0.012
t/mo

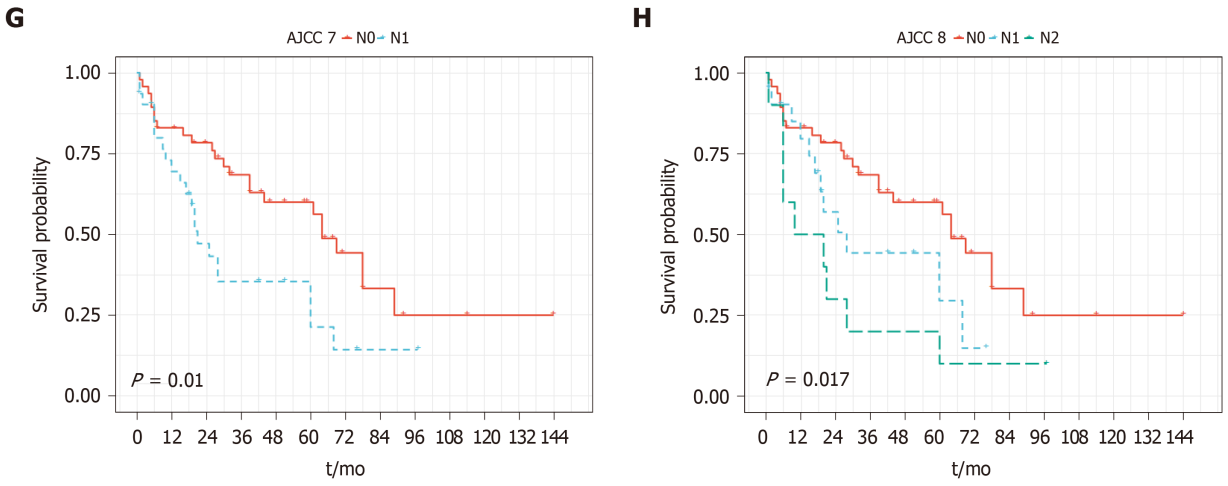


Figure 2 Kaplan-Meier curves for survival of the invasive intraductal papillary mucinous neoplasm. A: PFS stratified according to AJCC 7th edition T staging; B: PFS stratified according to the AJCC 8th edition T staging; C: PFS stratified according to AJCC 7th edition N staging; D: PFS stratified according to the AJCC 8th edition N staging; E: OS stratified according to AJCC 7th edition T staging; F: OS stratified according to the AJCC 8th edition T staging; G: OS stratified according to AJCC 7th edition N staging; H: OS stratified according to the AJCC 8th edition N staging. PFS: Progression-free survival; AJCC: American Joint Committee on Cancer; OS: Overall survival.

OS, although dividing N category into pN1 and pN2 in the 8th edition staging manual did not add further value in this group. The prognostic value of lymph node involvement has also been reported in a recent large scaled multi-institutional study. Morales-Oyarvide *et al*[20] demonstrated that the AJCC 8th edition staging system was a practical classification of lymph node involvement. Similar to other related validation studies[5,12-16], predominant patient population in this study (74%) had PDAC in the head of pancreas, and only 14% of the patients had PDAC in the tail of pancreas. Notably, the prognostic value of lymph node involvement was weaker in patients with resected distal pancreatic cancer[20].

This study has several strengths. It was a multicentered large-scale study designed to validate the major changes in the newer AJCC TNM staging system for resected distal pancreatic cancers. This study provides important insights for future revision of the AJCC staging system. Furthermore, all cases were re-staged according to the AJCC 7th or 8th edition and re-reviewed by pathologists subspecialized in gastrointestinal and pancreatic pathology. In addition, we included several potentially prognostic parameters such as radiographic evidence and histologic evidence of splenic vasculature and parenchymal invasion. In our study, we also compared the clinical behavior and risk stratification for invasive IPMN and non-IPMN associated PDAC.

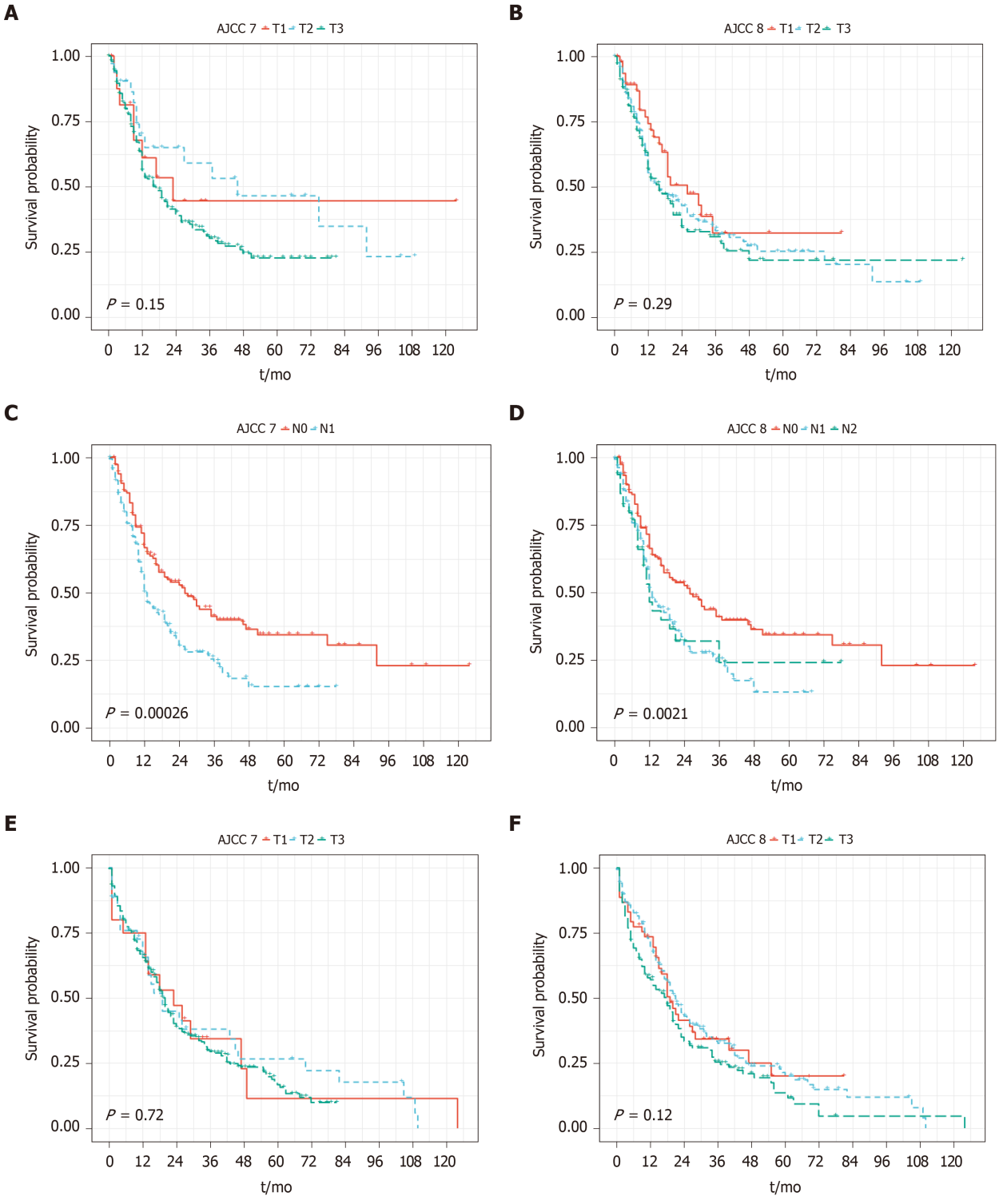
Our study also has some limitations. First, all cases were collected from major academic cancer centers that might have introduced selection bias. Second, the cases were collected from a 13-year period of time (2005-2018) during which multiple different AJCC staging editions (5th to 7th edition) had been applied for pancreatic cancer staging. However, all cases in this study were re-staged according to the 7th and 8th AJCC editions. The size of invasive IPMN was recorded from the surgical pathology report as this parameter is difficult to generate as it depends on the combination of macroscopic examination, sampling, and histology; histology review with measurement on slide alone does not provide an accurate assessment of this parameter.

In conclusion, our study demonstrates that the AJCC 8th edition TNM staging system provides even distribution for the T staging, however, it does not improve risk stratification when compared to previous staging system for resectable distal pancreatic cancers. Our study also demonstrates the significant difference of clinical outcome and risk stratification between invasive IPMN and non-IPMN associated PDAC. Our study indicates that tumor location and subtype are important factors to be considered in future revisions of the AJCC staging system for pancreatic cancer.

Table 3 Univariate and multivariable analysis for progression-free survival and overall survival in non-intraductal papillary mucinous neoplasm pancreatic ductal adenocarcinoma

Feature	Level	PFS		OS	
		HR (95%CI), *P* value			
Gender		0.89 (0.66-1.20), *P* = 0.43	-	1.05 (0.82-1.35), *P* = 0.69	-
Age		0.99 (0.98-1.01), *P* = 0.29	-	1.01 (1.00-1.03), *P* = 0.05	1.00 (0.98-1.02), *P* = 0.93
Tumor size		1.07 (0.99-1.15), *P* = 0.09	-	1.08 (1.02-1.16), *P* = 0.01	-
Tumor differentiation	Well	-	-	-	-
	Moderate	1.11 (0.64-1.91), *P* = 0.71	-	1.33 (0.78-2.27), *P* = 0.29	1.56 (0.65-3.78), *P* = 0.32
	Poor	1.21 (0.68-2.14), *P* = 0.52	-	1.72 (0.99-2.98), *P* = 0.05	2.23 (0.90-5.53), *P* = 0.08
Positive lymph node		1.06 (1.01-1.12), *P* = 0.03	-	1.08 (1.03-1.13), *P* < 0.01	-
T stage (AJCC 8th edition staging manual)	T1	-	-	-	-
	T2	1.39 (0.83-2.31), *P* = 0.21	1.22 (0.73-2.05), *P* = 0.45	0.97 (0.64-1.46), *P* = 0.87	1.12 (0.57-2.20), *P* = 0.73
	T3	1.56 (0.92-2.62), *P* = 0.10	1.19 (0.68-2.07), *P* = 0.54	1.33 (0.88-2.03), *P* = 0.18	0.80 (0.39-1.64), *P* = 0.54
N stage (AJCC 8th edition staging manual)	N0	-	-	-	-
	N1	1.73 (1.25-2.39), *P* < 0.01	1.59 (1.09-2.32), *P* = 0.02	1.75 (1.33-2.32), *P* < 0.01	2.54 (1.41-4.58), *P* < 0.01
	N2	1.57 (0.96-2.56), *P* = 0.07	1.47 (0.82-2.63), *P* = 0.20	1.83 (1.23-2.72), *P* < 0.01	2.45 (0.92-6.51), *P* = 0.07
Lymphovascular invasion		1.43 (1.05-1.95), *P* = 0.02	1.07 (0.73-1.56), *P* = 0.74	1.46 (1.12-1.89), *P* < 0.01	0.82 (0.45-1.49), *P* = 0.51
Perineural invasion		1.16 (0.76-1.78), *P* = 0.48	-	1.19 (0.83-1.73), *P* = 0.35	-
Splenic artery invasion		1.41 (0.71-2.80), *P* = 0.33	-	1.38 (0.81-2.36), *P* = 0.24	-
Splenic vein invasion		1.35 (0.82-2.23), *P* = 0.24	-	2.09 (1.44-3.03), *P* < 0.01	1.59 (0.83-3.02), *P* = 0.16
Positive margin		0.78 (0.43-1.40), *P* = 0.40	-	1.20 (0.78-1.82), *P* = 0.41	-
Splenic invasion		1.65 (0.95-2.85), *P* = 0.07	-	1.32 (0.82-2.11), *P* = 0.25	-
Adjuvant radiation therapy		0.93 (0.69-1.27), *P* = 0.66	-	0.85 (0.64-1.12), *P* = 0.25	-
Adjuvant chemotherapy		1.45 (0.90-2.34), *P* = 0.13	-	0.51 (0.37-0.71), *P* < 0.01	0.34 (0.20-0.58), *P* < 0.01
Radiographic splenic artery invasion		1.27 (0.87-1.84), *P* = 0.21	-	1.13 (0.81-1.59), *P* = 0.47	-
Radiographic splenic vein invasion		1.26 (0.88-1.80), *P* = 0.20	-	1.51 (1.10-2.07), *P* = 0.01	1.08 (0.65-1.80), *P* = 0.75

AJCC: American Joint Committee on Cancer; PFS: Progression-free survival; OS: Overall survival.


A
AJCC 7 T1 T2 T3
Survival probability
1.00
0.75
0.50
0.25
0.00
P = 0.15
0 12 24 36 48 60 72 84 96 108 120
t/mo
B
AJCC 8 T1 T2 T3
Survival probability
P = 0.29
t/mo
C
AJCC 7 N0 N1
Survival probability
P = 0.00026
t/mo
D
AJCC 8 N0 N1 N2
Survival probability
P = 0.0021
t/mo
E
AJCC 7 T1 T2 T3
Survival probability
P = 0.72
t/mo
F
AJCC 8 T1 T2 T3
Survival probability
P = 0.12
t/mo

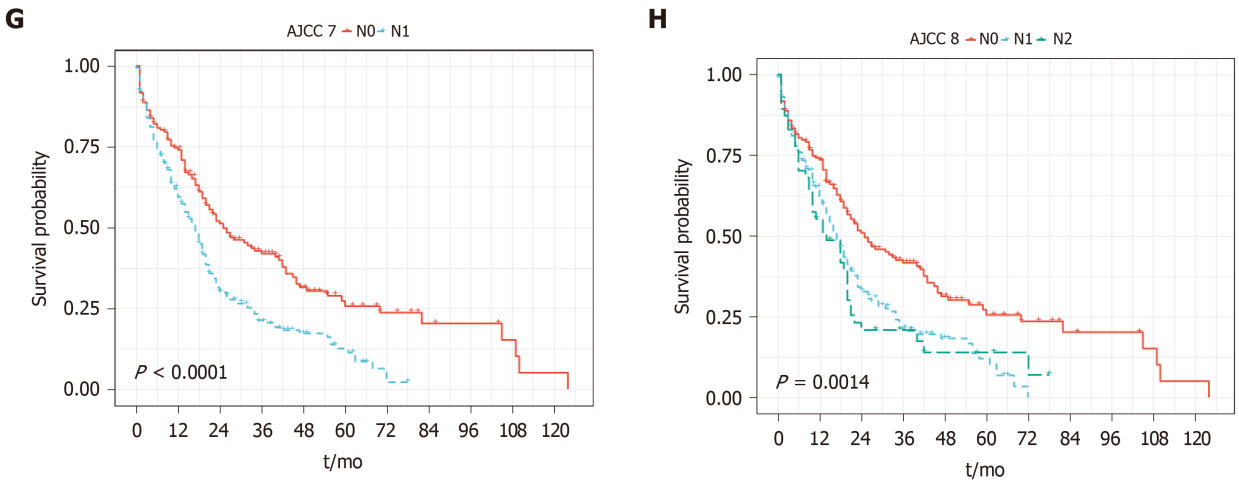


Figure 3 Kaplan-Meier curves for survival of the non-intraductal papillary mucinous neoplasm associated pancreatic ductal adenocarcinoma. A: PFS stratified according to AJCC 7th edition T staging; B: PFS stratified according to the AJCC 8th edition T staging; C: PFS stratified according to AJCC 7th edition N staging; D: PFS stratified according to the AJCC 8th edition N staging; E: OS stratified according to AJCC 7th edition T staging; F: OS stratified according to the AJCC 8th edition T staging; G: OS stratified according to AJCC 7th edition N staging; H: OS stratified according to the AJCC 8th edition N staging. PFS: Progression-free survival; AJCC: American Joint Committee on Cancer; OS: Overall survival.

ARTICLE HIGHLIGHTS

Research background

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer related death worldwide. For the purpose of better risk stratification and clinical management, the American Joint Committee on Cancer (AJCC) published the eighth edition staging manual for pancreatic cancer that has introduced significant changes for both tumor (T) staging and nodal (N) staging. Notably majority of the validation studies were focused on PDAC in the head of pancreas, and the resected distal pancreatic adenocarcinoma was likely underrepresented due to its clinical rarity. Whether the AJCC 8th edition staging manual provides equal risk stratification for both invasive intraductal papillary mucinous neoplasm (IPMN) and non-IPMN associated PDAC is also unclear.

Research motivation

It's important to investigate whether the new AJCC staging system provides risk stratification in patients with distal pancreatic cancers. It's also important to investigate the clinical behavior and risk stratification for invasive IPMN and non-IPMN associated PDAC.

Research objectives

This study aims to validate the AJCC 8th edition staging manual in distal PDAC.

Research methods

Clinicopathological data of resected distal PDAC cases were retrieved. All cases were re-staged based on the AJCC 7th and 8th edition, respectively. Categorical variables were compared with Fisher's exact test. Progression-free survival (PFS) and overall survival (OS) were evaluated through Kaplan-Meier curves and univariate/multivariate analyses.

Research results

T and N staging of both 7th and 8th edition sufficiently stratify PFS and OS in the entire cohort, although dividing into N1 and N2 according to the 8th edition does not show additional stratification. For PDAC arising in IPMN, T staging of the 7th edition and N1/N2 staging of the 8th edition appear to further stratify PFS and OS. For PDAC without an IPMN component, T staging from both versions fails to stratify PFS and OS.

Research conclusions

The AJCC 8th edition TNM staging system provides even distribution for the T staging, however, it does not provide better risk stratification than previous staging system for distal pancreatic cancer. There is significant difference of clinical outcome and risk stratification between invasive IPMN and non-IPMN associated PDAC.

Research perspectives

Tumor location and subtype are important factors to be considered in future revisions of the AJCC staging system for pancreatic cancer.

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The primary aim of the *World Journal of Gastrointestinal Pharmacology and Therapeutics* (*WJGPT*, *World J Gastrointest Pharmacol Ther*) is to provide scholars and readers from various fields of gastrointestinal pharmacology and therapeutics with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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ORIGINAL ARTICLE

Case Control Study

Shared changes in angiogenic factors across gastrointestinal vascular conditions: A pilot study

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Author contributions: Douglas AR performed the experiments and wrote the manuscript; Holleran G participated in sample collection; Smith SM served as scientific advisor and was involved in analysis; McNamara D designed the study; corrected the manuscript and served as the guarantor.

Institutional review board statement: Full ethical approval was obtained from the Tallaght Hospital/St James' Hospital Joint Research Ethics Committee.

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Abstract

BACKGROUND

Neovascularisation is common to a variety of gastrointestinal (GI) disorders with differing aetiologies and presentations; usually affecting adults above 60 years. Shared angiogenic factors modulated by disease specific elements could be a common denominator and represent novel diagnostic and therapeutic targets. As yet, assessment of angiogenic factors across several GI vascular disorders associated with recurrent bleeding and anaemia has not been reported.

AIM

To assess serum levels of angiogenic factors in several intestinal vascular disorders.

METHODS

A case control study was performed in Tallaght University Hospital in patients with endoscopically proven small bowel angiodysplasia (SBA), portal hypertensive gastropathy (PHG), gastric antral vascular ectasia (GAVE) and non-bleeding, non-anaemic controls. Using enzyme-linked immunosorbent assay, concentrations of Angiopoietin 1 (Ang-1), Ang-2 and vascular endothelial growth factor (VEGF) were measured from 2 serum tubes of blood following informed consent. The relative expression of Ang-1 and Ang-2 and Ang-1/2 ratio was calculated and compared between groups. Statistical analysis was applied using a *t*-test, and a P value of < 0.05 was considered significant.

RESULTS

To date 44 samples were tested: 10 SBA, 11 PHG, 8 GAVE and 15 controls. Mean age 60 (range 20-85) years and 20 (45%) were males. Controls were significantly younger (49 years *vs* 66 years, $P = 0.0005$). There was no difference in VEGF levels between the groups ($P = 0.6$). SBA, PHG and GAVE Ang-1 levels were similar and were significantly lower than controls, ($P = 0.0002$, 95%CI: 241 to 701). Ang-2 levels were statistically higher in PHG and GAVE groups compared to controls (P

= 0.01, 95%CI: 77.8 to 668) and as a result, also had a lower Ang-1/2 ratios compared to controls. While SBA Ang-2 levels were higher than controls, this did not reach statistical significance. Neither age nor haemoglobin level, which was similar between disease groups, could explain the difference. In addition, the median Ang-1/Ang-2 ratio for all patients was found to be significantly lower compared to controls, 8 *vs* 28 respectively, P = 0.001, 95%CI: -27.55 to -7.12.

CONCLUSION

Our novel pilot study suggests common alterations in Ang-1 and Ang-2 levels across several GI vascular disorders. Differences in Ang-1/Ang-2 ratios among vascular disorders compared to controls suggest disease-specific modulation.

Key words: Gastric antral vascular ectasia; Portal hypertensive gastropathy; Angiodysplasia; Angiopoietins; Angiogenic factors; Recurrent bleeding

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Core tip: This is the first study to look at key angiogenic factors across several distinct intestinal vascular disorders. Our novel study suggests a common alteration in Angiopoietin 1 (Ang-1) levels, a vascular factor associated with vessel stabilization and maturation, across a variety of gastrointestinal vascular disorders. VEGF appears not to play a significant role in these conditions. Serum elevation in Ang-2 levels and lower than normal Ang-1 levels are associated with clinically significant disease and warrant further investigation as potential biomarkers and therapeutic targets. This offers a potential final common pathway which could be of use diagnostically and therapeutically across several vascular conditions.

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INTRODUCTION

Vascular abnormalities can affect any part of the gastrointestinal (GI) tract and present with bleeding and anaemia[1]. Abnormal neovascularization, with friable, dilated superficial blood vessels, is common to a variety of GI disorders with differing etiologies and presentations including small bowel angiodysplasia (SBA), gastral antral vascular ectasia (GAVE) and portal hypertensive gastropathy (PHG)[2]. While associated with different clinical conditions and different locations these lesions are all common causes of recurrent or chronic intestinal bleeding especially in adults above 60 years. Reported rates of significant bleeding are; SBA (30%-50%), GAVE (2%-4%) and PHG (3%-26%)[3-5]. GI vascular malformations and bleeding in SBA have been associated with varying disturbances in angiogenesis, however, the precise mechanisms underlying these conditions remain unclear[3]. Our hypothesis is that localised aberrant neovascularisation develops in different conditions in response to a variety of upstream triggers including portal hypertension, hypoxia and inflammation, which may be regulated at a local level by common angiogenic regulators.

Angiogenesis is mediated by several angiogenic factors including the two major angiopoietin factors: Angiopoietin-1 (Ang-1) and Ang-2 and vascular endothelial growth factor (VEGF), a sub-family of the platelet-derived growth factor (PDGF). Ang-1 is involved in the maturation of blood vessels and has vascular protective properties, whereas Ang-2 promotes endothelial permeability. Both angiopoietins utilize their angiogenic regulatory effects via the tyrosine-kinase receptor (Tie-2)[6]. A more recent study has confirmed an imbalance in Ang-1/Ang-2 ratios in SBA patients with bleeding[3]. VEGF appears to play an essential role in promoting angiogenesis and vascular development; however, it can also cause vascular permeability and tissue oedema by working in synergy with Ang-2[2]. To date, the exact aetiology and pathophysiology of these conditions and the role of angiogenic factors is not yet fully

revised according to the STROBE Statement—checklist of items.

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established.

Due to a limited understanding of the role of angiogenic factors in common non-hereditary GI vascular disorders, no specific treatments targeting the angiogenic pathway are currently available. The management of these conditions is primarily endoscopic which can both diagnose and treat the source of bleeding. Despite this, these conditions frequently recur, and patients are often hospitalized for management of bleeding episodes, which has a significant health and financial impact. There is a need for identification of biomarkers as potential diagnostic and novel therapeutic targets, which could dramatically improve disease outcome for affected patients[7]. As yet, assessment of angiogenic factors across common GI vascular disorders has not been reported. Shared angiogenic factors modulated by disease specific elements could be a common denominator and represent novel diagnostic and therapeutic targets.

The aim of this study is to assess serum levels of angiogenic factors in different intestinal vascular disorders associated with recurrent bleeding and anaemia.

MATERIALS AND METHODS

Ethical approval was obtained from our institutions research and ethics committee and any patient over the age of 18 years undergoing an endoscopic procedure with a known diagnosis of SBA, PHG or GAVE and confirmed on endoscopy were invited to participate. Gender matched controls with negative surveillance colonoscopies, with two sequential negative faecal immunological tests were identified and invited to participate. Controls with significant dyspepsia, or evidence of GI bleeding or anaemia defined as haemoglobin (Hb) of < 11.5 g/dL in females and < 13 g/dL in males along with a serum ferritin of < 14, known chronic liver disease or non-liver related portal hypertension were excluded. Similarly, cases and controls with chronic renal failure, active malignancy or severe cardiac or respiratory failure were excluded. Recruitment continued until a minimum of 8 patients was included in each group.

Following informed consent, 2 serum separator tubes (3.5 mL each) of blood were taken on the same day using standard phlebotomy techniques. All relevant clinical data including basic demographics and relevant tests were recorded and filed on an encrypted database. Plasma samples were analysed routinely for Hb level and serum samples were left to clot for 30 min before undergoing centrifugation for 15 min at 1000 rpm. The resultant supernatant was extracted and stored in aliquots at -80 °C for batch analysis.

Serum levels of Ang-1, Ang-2 and VEGF were measured using commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (R and D systems, Minneapolis, MN, United States). Samples were prepared in duplicate and results were read at 450 nm absorbance. The intra-assay coefficients of variation (CV) were calculated as an average of all of the individual CVs for the sample concentration duplicates analysed by ELISA.

Statistical analysis

Categorical data was compared with a Chi^2 test. Serum levels of angiogenic factors were expressed as a mean and compared between groups using the Student *t*-test. Results were controlled for patient demographics including age, gender and haemoglobin level. In addition, the relative expression of Ang-1 and Ang-2, expressed as a ratio, per individual was calculated and compared between groups. A *P* value of < 0.05 was considered significant.

RESULTS

Population

Overall, 44 patients were recruited, 45% (n = 20) were males, with a mean age of 60 years (range 20-85), 15 controls, 10 with SBA, 11 PHG and 8 with GAVE. Subjects in the control group were significantly younger, 49 years (range 20-74) than cases, 65 years (range 38-85), P = 0.0005, 95%CI: 8 to 26. There was no difference in mean age among patient groups; 68 years (53-79) SBA, 60 years (38-81) PHG and 68 years (58-85) GAVE. There was no statistical difference in gender between controls and cases overall or by group with 47% (n = 7/15), 50% (n = 5/10), 27% (n = 3/11) and 50% (n = 4/8) being male respectively. A current haemoglobin level was available in 29 /44 (66%) subjects and was similar among cases. Mean Hb levels by group were; SBA 11.4 g/dL

(range 7.2-13.8), PHG 11.2 g/dL (9.5-12.2), GAVE 11.5 g/dL (8.3-15.3). While as expected the mean haemoglobin for controls was higher, this did not reach statistical significance 12.5g/dL (11.5 -13.2). However, more patients in the disease groups were anaemic compared to controls 1/5 (20%) *vs* 14/24 (59%), $P < 0.05$ (Table 1).

Serum levels of angiogenic factors

Ang-1/Ang-2: Mean Ang-1 levels were significantly higher in controls 53115 ± 17506 ng/mL than patients 29559 ± 18084, $P = 0.0002$, 95%CI: -35040 to -12072. While mean serum SBA, PHG and GAVE Ang-1 levels were all similar; 35696 ng/mL (range 12466-54338), 23111 ng/mL (range 1950-72445), 30753 ng/mL (range 9773-72609) respectively. As with Ang-1, mean Ang-2 levels were also similar among SBA, PHG and GAVE groups; 2803 ng/mL (range 125-13141), 4298 ng/mL (range 1299-8702) and 4232 ng/mL (range 1253-6081) respectively. Contrary to Ang-1 findings which were lower, the mean Ang-2 levels, 3764 ± 2763 ng/mL for patients were higher than controls, 1899 ± 772 ng/mL, $P < 0.01$, 95%CI: 389 to 3342.

The relative expression of Ang-1 and Ang-2 was calculated for each group. Controls as expected had a higher ratio; 35696/2803 ng/mL = 13 for SBA, 23111/4298 ng/mL = 5 for PHG, 30753/4232 ng/mL = 7 for GAVE *vs* 53114/1899 ng/mL = 28 for controls. However, the difference between SBA and control Ang-1/Ang-2 ratios did not reach statistical significance which was not unexpected as SBA Ang-2 levels while higher, was not statistically different from controls (Table 2).

In addition, the median Ang-1/Ang-2 ratio for all patients was found to be significantly lower compared to controls, 8 *vs* 28 respectively, $P = 0.001$, 95%CI: -27.55 to -7.12.

Age has been previously reported to affect angiopoietin levels[8]. In our cohort only SBA cases showed a variation in Ang-1 levels by age, with older age (> 70 years) associated with higher levels. Mean Ang-1 SBA < 70 years = 28306 ± 11479 ng/mL *vs* > 70 years = 46780 ± 5251 ng/mL, $P = 0.02$, 95%CI: 4142 to 32805. While mean Ang-1 levels by age for PHG and GAVE were 21661 ng/mL *vs* 24194 ng/mL, $P = 0.9$ and 29027 ng/mL *vs* 32415 ng/mL, $P = 0.8$ respectively. There was no difference in Ang-2 levels among cases by age. SBA 3562 ng/mL *vs* 1665 ng/mL, PHG 3555 ng/mL *vs* 3646 ng/mL and GAVE 4338 ng/mL *vs* 3055 ng/mL (Figure 1). Of note neither haemoglobin level or the presence of anaemia affected our findings.

VEGF: Again, mean serum VEGF levels were similar among cases; SBA = 443 ng/mL (range 273-964), PHG = 316 ng/mL (range 51-600) and GAVE = 435 ng/mL (range 288-637). Unlike angiopoietin levels, VEGF did not differ significantly between cases and controls. Mean VEGF for the control group was 421 ng/mL (range 250-678) (Table 2).

DISCUSSION

To our knowledge, this is the first study to look at key angiogenic factors across several distinct intestinal vascular disorders. In our pilot study subjects with endoscopically documented sporadic small bowel angiodysplasia, portal hypertensive gastropathy and gastric antral vascular ectasia all shared a common dysregulation in serum angiopoietin profile, with reduced levels of Ang-1 and higher levels of Ang-2 and resultant lower Ang-1/Ang-2 ratios. Adding weight to the hypothesis that dysregulated neovascularisation driven by Ang-2 excess is a key factor in the development of these conditions irrespective of upstream drivers. This offers a potential final common pathway which could be of use diagnostically and therapeutically across several vascular conditions.

Ang-2 is a circulating antagonist of the endothelial-specific Tie-2 receptor and has been identified as an important modulator of angiogenesis. While Ang-1 acts as a growth and maturation factor promoting development and stabilization of mature normal vessels in vivo by mediating endothelial cell interactions. Ang-2 antagonises Ang-1 and prevents Tie-2 receptor activation by competitively binding Tie-2. Ang-2 is known to be expressed at the site of vascular remodelling and its antagonism of Ang-1 actions therefore promotes vessel destabilization, possibly leading to the development of abnormal friable, leaky immature vessels, a hallmark of all these conditions[8,9].

Of interest, in contrast to previous studies which have documented elevated VEGF levels in GI disease and bleeding, VEGF levels were similar among controls and all disease groups in our cohort[10,11]. These findings mirror those we have previously demonstrated in a larger sporadic SBA cohort[8]. The role of VEGF and angiopoietins in blood vessel development and maturation is complex and their specific roles may

Table 1 Characteristics of study population

Factor	Controls	SBA	PHG	GAVE	Controls *vs* cases
Number	15	10	11	8	
mean age (range)	49 (20-74)	68 (53-79)	60 (38-81)	68 (58-85)	$P < 0.0005$
Male gender, *n* (%)	7 (47)	5 (50)	3 (27%)	5 (63)	NS
mean haemoglobin, g/dL	12.5	11.4	11.2	11.5	NS
Anaemia, *n* (%)[1]	1 (20)	4 (40)	6 (75)	3/5 (60)	$P < 0.05$

[1]Anaemia defined as Hb < 11.5 g/dL in females and < 13 g/dL in males. SBA: Small bowel angiodysplasia; PHG: Portal hypertensive gastropathy; GAVE: Gastric antral vascular ectasia; NS: Not significant.

Table 2 Levels of serum angiogenic factors according to group

	SBA	PHG	GAVE	Control
Ang-1 (ng/mL), mean (range)	35696 (12466-54338)	23111 (1950-72445)	30753 (9773-72609)	53115 (28624-82066)
P value *vs* controls	0.01	0.0003	0.01	
Ang-2 (ng/mL), mean (range)	2803 (125-13141)	4298 (1299-8702)	4232 (1253-6081)	1899 (742-3693)
P value *vs* controls	0.4	0.0008	0.003	
VEGF (ng/mL), mean (range)	443 (273-964)	316 (51-600)	435 (288-637)	421 (250-678)
P value *vs* controls	0.8	0.08	0.8	
Ang-1/Ang-2 ratios by group	13	5	7	28
Median Ang-1/Ang-2 ratios	8			28
P value *vs* controls	0.001			

SBA: Small bowel angiodysplasia; PHG: Portal hypertensive gastropathy; GAVE: Gastric antral vascular ectasia; VEGF: Vascular endothelial growth factor; Ang-1: Angiopoietin 1; Ang-2: Angiopoietin 2.

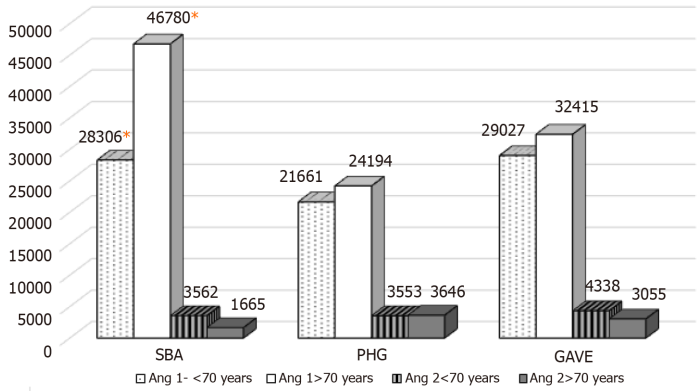


Figure 1 Mean angiopoietin 1 and angiopoietin 2 levels in ng/mL by age and group. *Statistically significant increase in Ang-1 in > 70 years $P = 0.02$, 95%CI: 4142 to 32805. SBA: Small bowel angiodysplasia; PHG: Portal hypertensive gastropathy; GAVE: Gastric antral vascular ectasia.

explain our findings. VEGF is a central mediator of early phase angiogenesis[12]. The initial phase of angiogenesis is characterized by VEGF-dependent formation and sprouting of blood vessels. Local concentrations of VEGF determine whether normal or aberrant angiogenesis is induced[13]. High local concentrations of VEGF result in vascular malformations and lesions resembling the chaotic architecture of angiodysplasias, thin-walled fragile vessels lacking smooth muscle cells that are susceptible to rupture[14,15]. To mature, primitive vascular complexes require remodelling during which vessels acquire a smooth muscle layer, and become less

permeable and friable, a process dependant on other angiogenic factors including Ang-1[16]. As such, it remains a possibility that high local tissue levels of VEGF are responsible for initiation of angiogenesis triggered by a variety of pathological processes. While high circulating levels of Ang-2 interferes with vessel remodelling and maturation, resulting in the abnormal friable, permeable vasculature, with a propensity for bleeding, common to these conditions. Confirmatory studies exploring local tissue VEGF expression would be required to confirm this hypothesis, which is beyond the scope of this pilot study.

While our initial results are of interest and warrant further investigation, there are significant limitations with our study. This is only a small, pilot study and findings will need to be confirmed in a larger cohort. Our sample size prevents effective subgroup analysis including the potential effects of age and other potential confounding factors including recent active bleeding events, although haemoglobin level did not affect expression in our cohort. We did control for gender and excluded conditions known to be associated with disturbances in angiogenic levels, which adds weight to our findings. In addition to a larger sample size, repeated testing at different times would be advantageous and help to establish whether these factors are predictive of disease severity, bleeding events and long-term outcomes. Similarly, our small cohort included patients with symptomatic disease only requiring endoscopic intervention which could represent a bias as potentially less severe forms may have a different angiogenic profile. However, clinical application is likely only to be relevant to just such a symptomatic cohort.

As mentioned previously, circulating angiogenic factors may not reflect local tissue activity and should be investigated to confirm the potential key role of angiopoietins and possibly VEGF across several GI vascular conditions. However, as a potential diagnostic or prognostic biomarker serum sampling is preferable and would be more useful clinically.

In conclusion, this is the first study to show common changes in angiogenic factors across several distinct intestinal vascular disorders. Serum elevation in Ang-2 levels and lower than normal Ang-1 levels are associated with clinically significant disease and warrant further investigation as potential biomarkers and therapeutic targets.

ARTICLE HIGHLIGHTS

Research background

Neovascularisation is a common feature of gastrointestinal **(**GI) vascular disorders with differing aetiologies and presentations; including small bowel angiodysplasia (SBA), gastral antral vascular ectasia and portal hypertensive gastropathy. These lesions are all common causes of recurrent or chronic intestinal bleeding especially in adults above 60 years. GI vascular malformations and bleeding in SBA have been associated with varying disturbances in angiogenesis, however, the precise mechanisms underlying these conditions remain unclear. Our hypothesis is that response to a variety of upstream triggers including portal hypertension, hypoxia and inflammation, which may be regulated at a local level by common angiogenic regulators including Angiopoietin 1 (Ang-1), Ang-2 and vascular endothelial growth factor (VEGF) may be responsible for these conditions.

Research motivation

At present, the precise mechanisms underlying these conditions remain unclear and assessment of angiogenic factors across several GI vascular disorders associated with recurrent bleeding and anaemia has not been reported. In addition, there is currently no specific treatment for these vascular conditions, the development of which is limited by a deficient knowledge of the underlying pathophysiology.

Research objectives

The overarching aim of our work is to identify angiogenic factors associated with the condition which may be useful both as diagnostic and prognostic markers and as future treatment targets.

Research methods

Using enzyme-linked immunosorbent assay, concentrations of Ang-1, Ang-2 and VEGF were measured from 2 serum tubes of blood using standard phlebotomy techniques. Categorical data was compared with a Chi^2 Test. Serum levels of

angiogenic factors were expressed as a mean and compared between groups using the Student *t*-test. Results were controlled for patient demographics including age, gender and haemoglobin level. A P value of < 0.05 was considered significant.

Research results

We observed a common reduction in Ang-1 levels and elevation in Ang-2 levels across several GI vascular disorders compared to controls. Differences in Ang-1/Ang-2 ratios among vascular disorders compared to controls suggest disease-specific modulation. This warrants further investigation as potential biomarkers and therapeutic targets.

Research conclusions

Our novel pilot study shows the common alteration in Ang-1 and Ang-2 levels across a variety of GI disorders. This suggests that the modulation of these angiogenic factors may play a vital role in these GI vascular conditions. This shows the value of these factors as potential biomarkers and therapeutic targets.

Research perspectives

Targeting these angiogenic factors could potentially serve as diagnostic or prognostic biomarkers and therapeutic targets in a clinical setting.

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ORIGINAL ARTICLE

Observational Study

Hepatobiliary manifestations in children with inflammatory bowel disease: A single-center experience in a low/middle income country

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Author contributions: El-Shabrawi MHF designed the study; Abou-Zekri M, Mogahed EA, Meshaal S and Tarek S participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Enayet A participated in statistical analysis and drafting the initial manuscript; El-Shabrawi MHF wrote and revised the article critically for important intellectual content.

Institutional review board statement: The study was reviewed and approved by the ethical committee in our Department of Paediatrics, Kasr Alainy School of Medicine, Cairo University, Cairo, Egypt.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

BACKGROUND

There has been a worldwide increase in the reported incidence of inflammatory bowel disease (IBD) in children over the past 2-3 decades. The hepatobiliary (HB) manifestations of IBD have been well-studied in children in industrialized and developed countries but are infrequently reported in low- and middle-income countries (LMIC) such as Egypt.

AIM

To determine the prevalence of the HB manifestations in a cohort of Egyptian children with IBD.

METHODS

This cross-sectional observational study was carried out over a period of 6 mo (between June 2013 to December 2013) at the Paediatric Hepatology and Gastroenterology Units of Cairo University Children's Hospital, which is the largest paediatric tertiary care centre in the country.

RESULTS

The study included 48 patients with confirmed IBD based upon clinical, laboratory, endoscopic and histopathological features, 29 (60.4%) were male. Twenty-four patients (50%) had ulcerative colitis (UC), 11 (22.9%) had Crohn's disease (CD) and 13 (27.1%) had unclassified-IBD (IBD-U), which was formerly known as indeterminate colitis. The mean age of the patients at the time of presentation was 8.14 (± SD 4.02) years and the mean age at the time of study enrolment was 10.16 (± SD 4.19) years. All patients were screened for HB manifestations by physical examination, liver function tests, imaging and liver

biopsy when indicated. HB disorders were confirmed in 13 patients (27.1%). Transaminases were elevated in 3 patients (6.3%). Two patients (4.2%) had elevated biliary enzymes (one was diagnosed as primary sclerosing cholangitis (PSC) and the other was diagnosed with PSC/autoimmune hepatitis overlap syndrome and the third patient had hepatitis C virus infection. Ten patients (20.8%) had bright echogenic liver on ultrasound suggesting fatty infiltration as a sequel of malnutrition or medication toxicity.

CONCLUSION

The commonest HB disorders in Egyptian children with IBD were abnormal liver function tests, fatty infiltration and PSC. These HB manifestations in paediatric patients in LMIC may be relatively more common than in industrialized countries. Therefore, IBD patients in LMIC should be meticulously screened for liver disease to allow prompt diagnosis and management.

Key words: Children; Crohn's disease; Egypt; Elevated liver enzymes; Hepatobiliary; Inflammatory bowel disease; Ulcerative colitis

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Core tip: The incidence of inflammatory bowel disease (IBD) in children has increased recently worldwide. Similarly, the reported incidence of the hepatobiliary (HB) manifestations of IBD in developed countries is rising, while in low- and middle-income countries, there are no much available reports especially in the paediatric age group. In this cohort of Egyptian children with IBD, all patients were screened for HB disorders by physical examination, liver function tests, imaging and liver biopsy when indicated. The most frequently reported HB disorders in these children with IBD were abnormal liver function tests, fatty infiltration and primary sclerosing cholangitis.

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INTRODUCTION

Inflammatory bowel disease (IBD) in childhood includes Crohn's disease (CD), ulcerative colitis (UC) and unclassified-IBD (IBD-U), which was formerly known as indeterminate colitis[1]. In the past few years, the incidence of IBD in children in industrialized and developed countries has increased[2-4]. However, although in the low- and middle-income countries (LMICs) there have been few reports, the incidence of IBD in children may also be increasing[5].

The characteristics of IBD in the Egyptian population are similar to the patterns seen in Asian and African populations, and the disease behaviour is milder than that reported in Western countries[6]. Extra-intestinal manifestations (EIMs) of IBD involving the skin, eyes and joints usually parallel the disease activity in the gut; however, diseases involving the hepatobiliary (HB) and pulmonary systems typically do not correlate with the disease activity of bowel inflammation[7]. The reported prevalence of liver and biliary tree disorders in IBD ranges from 3% to more than 50%, depending on whether the definition of the disease includes only definite and persistent conditions or transient alterations of liver function[8,9].

HB complications can be a result of the primary IBD process, medication toxicity, or due to an underlying primary hepatic disorder unrelated to IBD, such as hepatitis caused by viruses, drugs or toxins which are more prevalent in LMICs[10,11]. Some HB manifestations, such as cholelithiasis and portal vein thrombosis, can occur due to the effect of chronic inflammation and the severity of bowel disease with bouts of severe diarrhoea and dehydration[7]. Non-specific hepatomegaly is commonly reported in IBD and is strictly related to fatty infiltration in more than 30% of patients[12], and it does

report.

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not seem to be related to the patient's sex or type of IBD[8].

The management of these disorders often requires the expertise of a multidisciplinary team to achieve the best outcome[13].

The aim of the present study was to assess the prevalence and aetiology of HB manifestations in children and adolescents with IBD at a single centre in Egypt (which is classified as a LMIC) and compare them to those in some of the industrialized nations.

MATERIALS AND METHODS

Study design

This observational study was carried out at the Paediatric Hepatology and Gastroenterology Units of Cairo University Children's Hospital, Cairo, Egypt, over a 6-mo-period (between June 2013 and December 2013). Forty-eight children with an established diagnosis of IBD were included in the study. The diagnosis of IBD was based upon clinical, laboratory, endoscopic and histopathological features.

Ethical considerations

The study protocol was approved by the Research Ethics Committee of the Paediatrics Department, Faculty of Medicine, Cairo University, Egypt. The research was conducted in accordance to the Helsinki Declaration. All patients were enrolled in the study after an informed consent was obtained from their parents/guardians.

Participants

All patients < 18 years of age of both sexes with an established diagnosis of IBD were included. The clinical and laboratory data were retrospectively retrieved from 44 patients' files, and 4 newly diagnosed patients were prospectively enrolled during the study period. Patients were excluded if any crucial data was missing in their files.

Assessment and evaluation

Detailed history taking was carried out, including: (1) Age at the time of presentation; (2) Age at the time of IBD diagnosis; (3) Meticulous family history of IBD in other siblings or parents; (4) History of comorbid conditions other than IBD; (5) Symptoms suggestive of HB manifestations [jaundice, abdominal distension, pruritus, gastrointestinal (GI) bleeding]; (6) Age at the start of treatment and age at the time of onset of HB manifestations; and (7) Medication history [aminosalicylate, azathioprine, corticosteroids, methotrexate, infliximab and/or drugs for associated diseases such as colchicine for Familial Mediterranean fever (FMF)].

The patients' anthropometric measurements (weight and height) were plotted on Egyptian growth curves (Standard Egyptian Growth, 2008)[14], and the corresponding Z-scores were obtained.

All patients were subjected to meticulous clinical examination focusing on the signs of hepatic complications (jaundice, palmar erythema, spider nevi, lower limb oedema, and abdominal examination for organomegaly and ascites).

All patients were evaluated by liver chemistry, including the following: (1) Total and direct serum bilirubin; (2) Alanine aminotransferase (ALT); (3) Aspartate aminotransferase (AST); (4) Alkaline phosphatase (ALP); (5) Gamma glutamyl transpeptidase (GGT); (6) Serum albumin; and (7) Prothrombin time (PT) and concentration and international normalized ratio.

Abdominal ultrasound was performed in all patients using a greyscale device (FUKUDA-DENSHI; FF Sonic-400 Tokyo, Japan) to assess the liver size, texture, and echogenicity, dilatation of the intra-hepatic biliary radicals, gallbladder size, content and wall thickness, focal lesions, hepatic vasculature, and the presence of ascites. Splenic size was also assessed.

Serum immunoglobulin G (IgG), antinuclear antibody (ANA), smooth muscle antibody (SMA), anti-liver kidney microsomal 1, anti-neutrophil cytoplasmic antibody and anti-*Saccharomyces cerevisiae* antibody testing was performed in selected cases suspected of having autoimmune hepatitis (AIH) and/or primary sclerosing cholangitis (PSC). Viral markers were assessed for patients suspected of having hepatitis B virus (HBV) or hepatitis C virus (HCV) infections.

Diagnostic and follow-up upper and lower GI endoscopic examinations were performed for all patients to confirm the diagnosis of IBD and to monitor the treatment response. The gross endoscopic appearance as well as the histopathological

examination of mucosal biopsy specimens withdrawn at the time of GI endoscopy was the cornerstone for IBD diagnosis in our patients. Endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) was performed in cases suspected of having PSC or overlap syndrome (AIH and PSC).

Statistical analysis

The data were tabulated and statistically analysed. Qualitative data were expressed as numbers and percentages, and were compared by the chi-square test or Fisher's exact test when appropriate. Quantitative data were expressed as the mean ± SD, median and interquartile range, minimum and maximum, and were compared by the Student's *t*-test or the Mann-Whitney U test. In all tests, a probability (*P*) value was considered significant if less than 0.05.

RESULTS

Demographic characteristics

Based on the classic clinical findings, gross endoscopic appearance and microscopic histopathological features, we recruited 48 patients with IBD during this relatively short period from a large tertiary referral centre for all of Egypt. Twenty-nine (60.4%) patients were male; 24 (50%) had UC, 11 (22.9%) had CD, and 13 had IBD-U. The mean age at the time of presentation was 8.14 ± 4.02 years, while the mean age at the time of study enrolment was 10.16 ± 4.19 years.

The most common clinical presentation was recurrent abdominal pain in 47 patients (97.9%), followed by chronic diarrhoea with tenesmus and rectal bleeding (Table 1). None of our patients presented with ulcerating perianal disease. There was a positive family history of IBD in 9 patients (18.75%), representing other affected siblings. Twelve patients (25%) had other associated diseases: 11 had FMF, and 1 patient had systemic lupus erythematosus (SLE).

Regarding the symptoms suggestive of HB manifestations of IBD, 2 patients had jaundice associated with dark-coloured urine. Eighteen patients (37.5%) presented with abdominal distension, which may also have been attributed to IBD itself. One of our patients presented with pruritus, and none had manifestations of portal hypertension. Upper GI endoscopy confirmed the absence of oesophageal varices. The mean age at the onset of hepatic complications in our patients diagnosed with IBD was 12.10 ± 3.51 years.

The median (range) weight and height by Z score were -1.0 (-5.0-3.0) and -1.2 (-6.6-1.7), respectively. Sixteen patients (33.4%) were on the 3rd percentile or lower in weight, while 13 (27.1%) had short stature.

Abdominal examination showed that 16 patients (33.3%) had abdominal distension, 6 of whom (12.5%) had only hepatomegaly, with mean ± SD size of the liver in the right midclavicular line and midline of 4.83 ± 1.72 and 5.33 ± 2.94 cm, respectively. Two patients (4.2%) had splenomegaly and none had ascites.

With regard to administered medications for the treatment of IBD at the time of study enrolment: (1) Thirty-five patients (72.9%) were receiving mesalamine; (2) Twelve (25%) received salazopyrine; (3) Twenty-six patients (54.2%) were receiving corticosteroids; (4) Twenty patients (41.7%) received azathioprine; (5) Four patients (8.35%) received methotrexate; and (6) Only one patient received infliximab.

In addition, 11 patients (22.9%) who were diagnosed with FMF received colchicine.

Outcome data

Liver chemistry revealed that 3 patients (6.25%) had elevated liver enzymes, 2 patients (4.2%) had direct hyperbilirubinaemia, and 10 patients (20.8%) had hypoalbuminaemia that may also have been attributed to IBD, as none of the patients had coagulopathy (Table 2). Abdominal ultrasonography revealed that 10 patients (22.7%) had a bright echogenic liver (Figure 1), which may suggest fatty infiltration. Two patients (4.5%) had dilated intrahepatic biliary radicals. Two patients (4.5%) had thickened gallbladder walls, while 4 patients (9.1%) had splenomegaly. None had ascites.

Therefore, the overall frequency of HB manifestations in this study was 27.1% (13 patients). Of these 13 patients, 2 had cholestatic jaundice, elevated transaminases and elevated ALP and GGT, and both were confirmed to have PSC by MRCP (Figure 2) and percutaneous liver biopsy (Figure 3). One of these 2 patients had elevated serum IgG levels, positive ANA and positive SMA, and was diagnosed with PSC/AIH overlap syndrome. Another patient with elevated liver enzymes was diagnosed with

Table 1 The presenting symptoms of children with inflammatory bowel disease in the study group (*n* = 48)

Complaint	*n*	Percentage
Abdominal pain	47	97.9
Diarrhoea	43	89.6
Tenesmus	36	75
Bleeding per rectum	35	72.9
Chronic fatigue	19	39.6
Weight loss	13	27.1
Low grade fever	13	27.1

Table 2 The liver biochemical profile of children with inflammatory bowel disease in the study group

Parameter	Result	Range
ALT in median (IQR) (up to 45 U/L)	16.5 (13-24.8)	8-184
AST in median (IQR) (up to 75 U/L)	27 (20.5-34)	6-226
TB in median (IQR) (up to 1.4 mg/dL)	0.4 (0.3-0.5)	0.1-3.4
DB in median (IQR) (up to 1.4 mg/dL)	0.1 (0.1-0.1)	0-1.7
ALP in median (IQR) (up to 640 U/L)	161 (88-239)	50-439
GGT in median (IQR) (up to 50 U/L)	15 (12.3-19.8)	8-331
Albumin in mean ± SD (3.5-4.5 g/dL)	3.75 ± 0.78	1.3-5.3
PT in mean ± SD (s)	13.1 ± 0.59	11.7-14.8
PC in mean ± SD	92.8 ± 7.41	76-100
INR in mean ± SD	1.02 ± 0.05	1-1.2

ALT: Alanine aminotransferase; AST: aspartate aminotransferase; ALP: Alkaline phosphatase; DB: Direct bilirubin; IQR: interquartile range; GGT: Gamma-Glutamyl transferase; INR: International normalized ratio; PC: Prothrombin concentration; PT: Prothrombin time; TB: Total bilirubin.



Figure 1 Abdominal ultrasound of a patient with inflammatory bowel disease showing increased liver echogenicity.

SLE and had a concomitant HCV infection. He was recently treated for HCV with oral direct acting antivirals (sofosbuvir/ledipasvir) and achieved sustained virological response followed by normalization of his liver functions. Ten patients had echogenic liver by ultrasound: 3 of them were receiving steroids, 3 patients had malnutrition with poor growth, and 4 patients had both risk factors.

Regarding the 6 patients (12.5%) who had hepatomegaly, two had PSC, 3 had fatty liver, and the remaining patient had HCV infection.



Figure 2 Magnetic resonance cholangiopancreatography of a patient with inflammatory bowel disease and primary sclerosing cholangitis showing dilatation of the intra- and extra-hepatic biliary tree with multiple strictures (arrows).

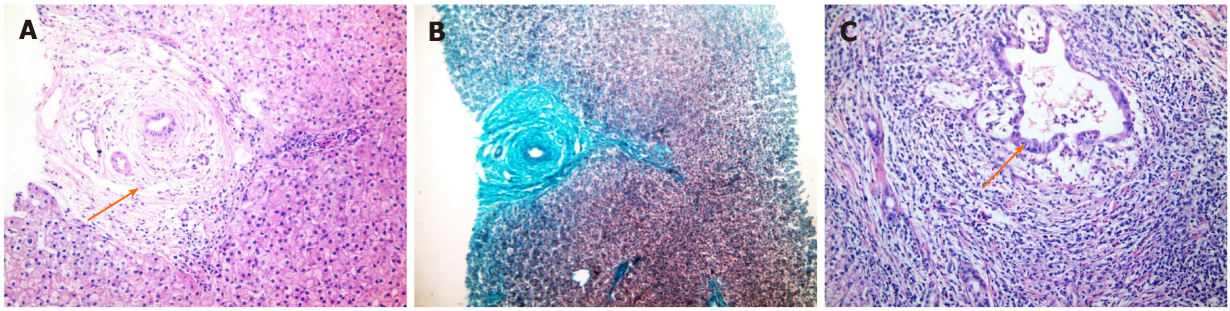


Figure 3 Histopathological findings of a liver biopsy specimen from a patient with inflammatory bowel disease showing the features of primary sclerosing cholangitis. A: Diffuse bile ductular proliferation "Onion skin" fibrosis around affected ducts, concentric collagen fibre deposition; B: Same findings by Masson's trichrome stain which imparts a blue color to type 1 collagen against a red background of hepatocytes; C: Aggression of inflammatory cells towards the biliary epithelium and the portal area is markedly expanded with lymphoplasmacytic inflammatory cellular infiltrate (all photos H&E, Original magnification × 400).

DISCUSSION

IBDs, including CD, UC, and IBD-U, are chronic and relapsing intestinal disorders[15]. In the Asia–Pacific region, a marked increase in the number of cases of IBD was found, which was compatible with a similar trend worldwide[16-18]. Our current study included 48 Egyptian children with IBD. The most common clinical presentation in our patients was recurrent abdominal pain, which was present in 97.9% of patients, followed by chronic diarrhoea. These findings are consistent with those of other studies where the most common presenting symptoms of IBD in children were abdominal pain (87.5%) and diarrhoea (75.0%)[14].

A positive family history remains the strongest recognizable risk factor for the development of IBD and is reported in approximately 8%-12% of IBD patients[19]. Genetic factors, such as nucleotide-binding oligomerization domain containing 2 (NOD2) and other autophagy-related genes, such as ATG16L1 (autophagy-related 16-like 1), have been implicated in the aetiopathogenesis of IBD[20]. NOD2, a cytosolic protein expressed in monocytes, functions as an intracellular receptor for a bacterial product and is also a component of the innate immune system. CD is associated with frameshift mutations in NOD2, resulting in a truncated and non-functioning protein[21]. Autophagy, the major lysosomal pathway for degrading and recycling cytoplasmic material, constitutes an important homeostatic cellular process. Of interest, single-nucleotide polymorphisms of ATG16L1, a key component in the autophagic response to invading pathogens), have been associated with an increased risk of developing CD[22]. In the current study, nine patients had a positive history of IBD in their siblings. This relatively higher prevalence of positive family history in our study is mostly related to the deeply rooted Egyptian cultural traditions of consanguineous marriages and a large family size[23]. Therefore, we recommend meticulous genetic studies in our patients in Egypt and other LMICs who have a high prevalence of consanguinity.

The most common extra-intestinal manifestation of IBDs, particularly CD, in childhood and adolescence is reported to be impaired physical growth[24]. This was found in the present study, where almost one-third of our patients was on the 3rd percentile or lower for body weight, while 27.1% had short stature[25].

In the present study, 11 patients (22.9%) were also diagnosed with FMF confirmed by genetic testing. This observation agrees with the study by Salah *et al*[26] conducted at our institute, which reported a significant association between *MEFV* gene mutation and IBD in Egyptian children, especially in patients with indeterminate colitis (IBD-U)[26].

HB disorders are common in patients with IBD. Persistently elevated transaminases are observed in approximately 20%-30% of these patients[10]. An Italian study reported that abnormal liver chemistry was detected in 20.9% of IBD patients[12]. In this study, the frequency of overall HB manifestations was 27.1% (13 patients). Liver chemistry revealed that only 3 patients (6.25%) had elevated liver enzymes. Of the two patients who had cholestatic jaundice, one was diagnosed with PSC, and the other was diagnosed with PSC/AIH overlap syndrome. This finding was similar to that reported by Broomé *et al*[27], who found that 1.4%-7.5% of patients with IBD eventually developed PSC during the course of their disease. Patients with PSC usually present with jaundice[28], which was the case in our patients. The disease onset of PSC is typically insidious, but it has been increasingly diagnosed at the asymptomatic stage, likely because of the widespread availability of ERCP and MRCP for evaluating elevated serum ALP levels[10].

AIH/PSC overlap must be considered in any patient with IBD, particularly UC, and PSC exhibits elevated transaminases, polyclonal hyper-gamma-globulinaemia and a liver biopsy suggestive of periportal hepatitis[28]. Autoimmune liver disease is reported in up to 7.8% of children with IBD[29]. In the present study, only one patient suffered from AIH/PSC overlap syndrome.

Corticosteroids may alter the hepatic lipid metabolism and induce hepatic steatosis[30]. Malnutrition is another factor that is highly associated with hepatocyte fatty infiltration. Furthermore, protein synthesis is decreased during protein-calorie malnutrition, which plays a role in triglyceride export into the blood[31]. Thus, triglycerides are retained within the hepatocytes resulting in fatty liver. Increased import of free fatty acids into the liver as a result of abdominal lipolysis, which develops during the weight-loss stages, may add to this process[32]. This finding is in agreement with our study, in which 10 patients had echogenic liver by ultrasound; 3 of them were receiving steroids, 3 patients had malnutrition with poor growth according to the Egyptian growth curves[25], and 4 patients had both risk factors. Although the diagnosis of fatty liver needs to be confirmed histologically, screening can be accomplished *via* analysis of serum aminotransferases, GGT, triglyceride levels and ultrasonographic appearance of a bright echotexture of the liver[8]. On the other hand, a previous study suggested a complex, multifactorial relationship between the IBDs and the development of non-alcoholic fatty liver disease (NAFLD) beyond the scope of current pharmacological intervention[33].

As immunosuppressive drugs are being used more frequently in IBD, concerns about viral reactivation are increasing. The impact of immunosuppressive therapy for IBD on HCV remains controversial, as steroids can promote viral replication. Egypt has the highest prevalence of HCV infection in the world[34]. The prevalence of HCV infection has increased in IBD patients in comparison to the general population both in Spain and France in studies over the last decade of the 20th century[35]. Similarly, one of the patients (2%) in the current study had associated chronic HCV infection. This patient had multiple risk factors for HCV acquisition, including blood transfusion, recurrent hospitalizations, and repeated endoscopies with intestinal biopsies. However, other studies have demonstrated a similar prevalence in the general population[36,37].

The drug armamentarium for IBD is very wide and might be frequently complicated by hepatotoxicity, adding to the spectrum of liver disease in IBD. However, drug therapy for the associated liver disease itself might be limited, and appropriate timely diagnosis might be all that is offered to the patients, but that issue is beyond the scope of this study.

The prognosis of IBD patients with liver disease is worsening, and close collaboration between IBD clinicians, paediatric hepatologists/gastroenterologists, and primary care providers should be encouraged for early diagnosis, shared decision making and optimal outcome before end-stage liver disease sets in[10]. Liver transplantation (LTx) is not readily available in LMICs. Moreover, recipients with comorbidities at the time of LTx, including those with IBD, have increased morbidity and mortality. Due to the association between IBD and PSC, the frequency of LTx in

patients with IBD has increased[10]. Patients with a longer duration of UC and extensive colonic involvement are known to have an increased risk of colorectal cancer. Routine colorectal cancer surveillance should continue for patients with IBD who have had LTx, even in the setting of clinically quiescent disease[37].

In conclusion, the prevalence of HB manifestations in paediatric patients with IBD in Egypt, as one of the LMICs, is relatively high compared to industrialized countries. Investment in research for confounding factors in LMICs might be justifiable, including family genetic studies that might diagnose early cases of IBD and work-up for very early-onset IBD. These patients should be screened for liver disease to allow prompt diagnosis and the offer of available treatment. The most common HB disorders in children with IBD are abnormal liver tests, fatty infiltration and PSC.

Suggestion for further research

Screening for HB manifestations of IBD is mandatory, as many of these complications could be initially asymptomatic. HBV and HCV should not be overlooked and should be screened for in patients with IBD, as these patients have multiple risk factors for HCV acquisition including endoscopies, blood transfusion and recurrent hospitalizations. Therefore, it is necessary to implement mass screening for HCV in these high-risk groups especially in an endemic country such as Egypt. Health education for parents and patients should be carried out to increase knowledge on the nature of the disease and treatment options. Genetic research on the diagnosis of very early-onset IBD and family screening should be emphasized.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a systemic illness that can present with multiple extra-intestinal manifestations. Hepatobiliary (HB) disorders associated with IBD are not uncommon. From an aetiopathogenic perspective, there are multiple layers of evidence suggesting that these disorders are not isolated entities and may share mechanistic pathways. All of these disorders usually manifest with abnormal hepatic biochemical tests. Of the HB complications of IBD, primary sclerosing cholangitis (PSC) carries the most significant clinical implications and remains a highly challenging disease to manage.

Research motivation

Recently, the reported incidence of IBD in paediatrics has increased dramatically. The HB manifestations of IBD have been well studied in children in the industrialized and developed countries. On the other hand, there is a paucity of studies on IBD in low- and middle-income countries (LMICs) such as Egypt.

Research objectives

The main objective was to determine the frequency of HB manifestations in Egyptian paediatric patients with IBDs, to achieve an early diagnosis in order to obtain prompt treatment. Moreover, the risk factors associated with the occurrence of HB complications in patients with IBD were determined.

Research methods

This study was carried out at the Paediatric Hepatology and Gastroenterology Units of Cairo University Children's Hospital over a 6-mo-period. Patients younger than 18 years of age of both sexes were included. The available data were retrieved from the files of patients diagnosed with IBD as well as newly diagnosed patients. The collected data included a full medical history such as name, age, sex, residence, meticulous family history of IBD and other associated autoimmune illnesses; history of jaundice; abdominal distention; pruritus; bleeding; a history of blood transfusion; and a detailed medication history. A meticulous clinical examination was performed, including the assessment of anthropometric measures and a general and detailed abdominal examination. Laboratory investigations, such as CBC, complete liver functions (ALT, AST, total and direct bilirubin, ALP, GGT, albumin and PT) were performed in all patients. In addition, serum immunoglobulin G, antinuclear antibody, smooth muscle antibody, anti-liver kidney microsomal 1, anti-neutrophil cytoplasmic antibody and anti-*Saccharomyces cerevisiae* antibody were performed for selected cases suspected of having autoimmune hepatitis (AIH) and/or PSC. Viral markers were assessed in

patients suspected of having hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. Abdominal ultrasound was performed in all patients. Endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography was performed for cases suspected of having PSC. Percutaneous liver biopsy was performed when indicated.

Research results

We recruited 48 paediatric patients with IBD. Twenty-nine (60.4%) patients were male. Twenty-four (50%) had UC, 11 (22.9%) had CD, and 13 patients had IBD-U. The median (range) of the weight and height by Z score were -1.0 (-5.0-3.0) and -1.2 (-6.6-1.7), respectively. Sixteen patients (33.4%) were on the 3rd percentile or lower for weight, while 13 (27.1%) had short stature. The most common clinical presentation was recurrent abdominal pain, which was present in 47 patients (97.9%), followed by chronic diarrhoea with tenesmus and rectal bleeding. None of our patients presented with ulcerating perianal disease. There was a positive family history of IBD in 9 patients (18.75%). Twelve patients (25%) had other associated diseases: 11 had FMF, and one patient had systemic lupus erythematosus. Therefore, the overall frequency of HB disorders in this study was 27.1% (13 patients). Two patients had cholestatic jaundice, one of them was diagnosed with PSC, and the other patient had PSC/AIH overlap syndrome. Three (6.3%) patients had elevated liver enzymes. Ten (20.8%) patients had echogenic liver suggesting fatty changes.

Research conclusions

HB manifestations in paediatric patients with IBD in LMICs are more common than those in the industrialized countries. Investment in research for confounding factors in LMICs is cost-effective, including family genetic studies that can offer an early diagnosis of IBDs in general and specifically for very early-onset IBD. The importance of awareness of the implications and causes of abnormal hepatic biochemical tests in IBD patients is due to the wide range of possible complications and risks associated with the medications used to treat and manage them. Hepatic biochemical tests should be routinely performed in these patients. When abnormalities are detected, a prompt step by step diagnostic approach should be followed until an aetiology is reached. The frequency of HB manifestations in our patients was not low and may be affected by the type of treatment modality and malnutrition. The highest proportion was found in those suffering from an echogenic liver by abdominal ultrasound, and many of these patients received corticosteroids.

Research perspectives

Screening for HB manifestations of IBD is mandatory, as many of these complications may be initially asymptomatic. Complete liver function tests and abdominal ultrasound should be performed on a regular basis. A high index of suspicion of PSC should be raised in patients who present with an unexplained cholestatic jaundice or elevated ALP levels. An echogenic liver detected by abdominal ultrasound requires thorough assessment of the patient's growth and medication history. Liver biopsy may be needed in selected cases for the definitive diagnosis of NAFLD. Drugs used to treat IBD should be administered cautiously, as a majority of them can potentially cause hepatotoxicity. HBV and HCV should be screened for in patients with IBD especially in endemic areas such as Egypt. Patients with IBD have multiple risk factors for HCV and HBV acquisition, including repeated endoscopies, transfusion of blood products and recurrent hospitalizations. Genetic research should include the diagnosis of very early-onset IBD and screening of similar cases in families.

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ORIGINAL ARTICLE

Prospective Study

Tongue thickness in health vs cirrhosis of the liver: Prospective observational study

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Author contributions: Tandon M conceived and wrote the manuscript and performed the literature search and review; Sigh H collected and compiled data and reviewed the literature; Singla N collected L3 skeletal muscle index data, trained others on ultrasonography measurements, and supervised ultrasonography measurements; Jain P performed statistical analysis of the data; Pandey CK reviewed the manuscript, and provided intellectual input.

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Clinical trial registration statement: The study titled "Study of thickness of tongue by ultrasound and its relation with severity of disease in patients with cirrhosis of liver: Prospective Study", is registered with clinical trials registry of India vide No.

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Abstract

BACKGROUND

Malnutrition affects 40%-90% of patients with cirrhosis of the liver. L3 skeletal muscle index (L3SMI) is presently accepted as the most objective and quantitative measure available for sarcopenia, a surrogate marker of malnutrition. L3SMI application is, however, limited by non-availability of computed tomography scanning in remote areas, cost, need for extensive training, and the risk of exposure to radiation. Therefore, an alternative dependable measure with wider availability is needed. Malnutrition causes sarcopenia not only in skeletal muscles but also in other muscular structures such as the psoas muscle, diaphragm and tongue. We therefore hypothesised that the tongue, being easily accessible for inspection and for measurement of thickness using ultrasonography, may be used to document sarcopenia.

AIM

To measure and compare tongue thickness in healthy individuals and in patients with cirrhosis of the liver and to study its correlation with conventional prognostic scores for patients with cirrhosis of the liver.

METHODS

Tongue thickness was measured using ultrasonography. One hundred twenty subjects of either gender aged 18 to 65 years were studied, with 30 subjects in each group. The tongue thickness was compared between groups based on "Child Turcotte Pugh" (CTP) scores. The correlations between measured tongue thickness and "Model for end stage liver disease" (MELD) score and between age

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and measured tongue thickness were also assessed.

RESULTS

Mean tongue thickness (mean ± SD) in patients with CTP class A, B and C was 4.39 ± 0.39 cm, 4.19 ± 0.53 cm, and 3.87 ± 0.42, respectively, and was 4.33 ± 0.49 cm in normal healthy individuals. Significant differences were seen in tongue thickness between patients with CTP class C and those with CTP class A and B ($P < 0.05$). Patients with CTP class C also had a significantly reduced tongue thickness than normal individuals ($P < 0.05$). However, no significant difference was seen in tongue thickness between patients with CTP class A and B and normal individuals. A statistically significant, negative correlation was found between MELD score and tongue thickness ($r = -0.331$) ($P < 0.001$). No correlation was observed between L3SMI and MELD score ($r = 0.074$, $P = 0.424$). L3SMI (mean ± SD) in healthy subjects was 39.66 ± 6.8 and was 38.26 ± 8.88 in patients with CTP class C, and the difference was not significant. No significant correlation was found between age of the patients and tongue thickness. Intra-class correlation coefficient was used to determine the reliability of the tongue thickness measurements. The intra-class correlation coefficient was 0.984 (95%CI: 0.979-0.989) and was indicative of good reliability.

CONCLUSION

Tongue thickness measured by ultrasonography, correlates significantly with the severity of liver disease, as assessed by CTP and MELD scores. The patients with a CTP score ≥ 10 have significantly reduced tongue thickness as compared to normal individuals and those with less severe liver disease and CTP scores of 5-9. No significant difference in tongue thickness was found between healthy individuals and CTP class A and B patients.

Key words: Sarcopenia; Malnutrition; Cirrhosis of the liver; Child Turcotte Pugh class; Model for end stage liver disease score; Ultrasonography

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Core tip: Sarcopenia has implications for the management and outcome of patients with cirrhosis of the liver and is therefore included in prognostication. However, the only objective and reproducible measure for sarcopenia using computed tomography (CT) scanning which measures skeletal muscle thickness, is the L3 skeletal muscle index (L3SMI). However, the application of L3SMI is restricted by the need for CT scanning. Compared with CT scan measured L3SMI, tongue thickness can easily be measured in a reproducible manner and accurately with minimal training using ultrasonography and as suggested by this study, is a more sensitive indicator of sarcopenia. Further studies are required to validate these findings and propose tongue thickness as a tool to diagnose sarcopenia.

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INTRODUCTION

Malnutrition has been estimated to affect 50%-90% of patients with cirrhosis of the liver[1]. However, malnutrition is frequently overlooked, in part because nutritional assessment can be difficult in patients with cirrhosis due to fluid retention and because patients with cirrhosis may develop simultaneous loss of skeletal muscle and gain of adipose tissue, culminating in the condition of "sarcopenic obesity"[1]. Sarcopenia is characterised by the loss of muscle mass and is a surrogate marker of malnutrition[2]. Severity of liver disease is assessed using "Model for end stage liver disease" (MELD)

score, which is calculated by an online calculator using values for serum creatinine, bilirubin and the international normalised value for prothrombin time. The MELD score is also used to decide upon organ allocation for liver transplant[3], and currently does not include a measure of sarcopenia. Researchers have suggested changes in the MELD score calculation to include a measure of sarcopenia, considering its prognostic implications[4]. Similarly, the "Child Turcotte Pugh" (CTP) score, a conventional scoring system for severity of liver disease based on values of serum bilirubin, albumin, international normalised value for prothrombin time, and measures of encephalopathy and ascites, do not include any measure of sarcopenia[5]. L3 skeletal muscle index (L3SMI) is presently accepted as the most objective and quantitative measure of sarcopenia[6]. However, non-availability of computed tomography (CT) scanning in remote areas, cost, and exposure to radiation are limitations to the use of L3SMI; besides, the use of CT for documenting and quantifying sarcopenia can be justified only in patients who have an indication for CT as part of their standard medical care due to risks of radiation exposure. An optimal index for sarcopenia in terms of availability, reproducibility, practicality, and of prognostic significance is therefore needed and remains a challenging issue.

Malnutrition causes sarcopenia in several muscular structures besides the much-studied lumbar muscles, such as the diaphragm, psoas muscle and tongue[7-9]. In the quest for ultrasonography (USG)-based, bedside targets for documenting sarcopenia, we hypothesised that the tongue being a muscular structure affected by sarcopenia with easy access for inspection and measurement, may also be used to quantify and document sarcopenia. Therefore, a prospective study was conducted with the primary objective to measure and compare tongue thickness in healthy individuals and in patients with cirrhosis of the liver. The secondary objective was to determine the correlation between tongue thickness and conventional prognostic scores for patients with cirrhosis of the liver.

MATERIALS AND METHODS

The study was performed at a tertiary care institution after approval by the institutional ethics committee. Consent for the study protocol was obtained from the study subjects. To study 30% difference with power of 80 and type II error of 5%, we needed to study 30 subjects in each group. A total of 120 subjects, who satisfied the inclusion criteria were enrolled and studied from May 2017 to October 2018.

Patients with cirrhosis of the liver due to any aetiology and healthy individuals aged 18-65 years and a body mass index (BMI) > 18 and < 30, visiting the hospital for reasons other than illness, were included in the study. Individuals aged less than 18 years and more than 65 years, patients with acute liver failure and those with glossitis were not included in the study. Tongue thickness was measured using USG with a 3.75 MHz convex probe while the subjects were seated in an upright position. The subjects were instructed to swallow their saliva several times to set the tongue at the resting position, and then the ultrasonic measurements were carried out. The measurement points were determined on the upper and lower surfaces of the lingual muscles in the centre of the plane, perpendicular to the 'Frankfurt horizontal plane' in a frontal section (Figure 1). The Frankfurt horizontal plane is formed by drawing a straight horizontal line from the top of the ear canal to the bottom border of the eye along either side of the human skull. This line is called the Frankfurt horizontal line[10]. The vertical distance was measured from the surface of the mylohyoid muscle to the tongue dorsum (Figure 2). Measurements were performed thrice in freeze-frame when the tongue was restored to the resting position after swallowing saliva, and the mean values were obtained. Tongue thickness was measured for all the study subjects, but the L3SMI was calculated from CT scans only for patients with CTP class C who were being investigated for liver transplant and for healthy individuals who were evaluated as possible organ donors. MELD scores were calculated using the online calculator at https://www.mdcalc.com/meld-score-model-end-stage-liver-disease-12-older. Tongue thickness was compared between the groups based on CTP scores. Correlations were also determined between measured tongue thickness and MELD score, and between age and measured tongue thickness.

Statistical analysis

Data are presented as mean ± SD or frequencies (percentage) and were analysed using SPSS 23.0 software. One-way ANOVA was used to test the significance of parametric data and the Kruskal-Wallis test for non-parametric data. Comparison of categorical

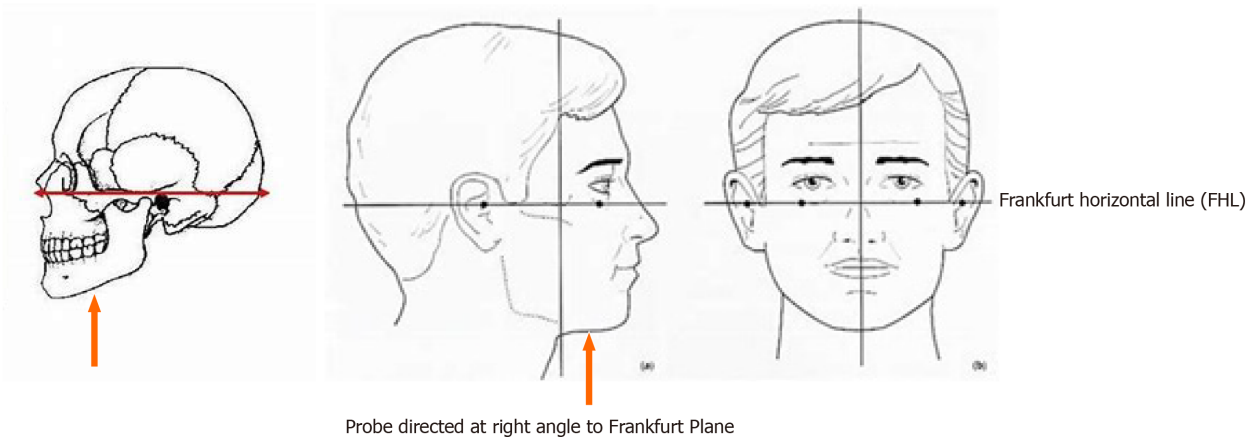


Figure 1 Ultrasonography probe position for measuring tongue thickness.

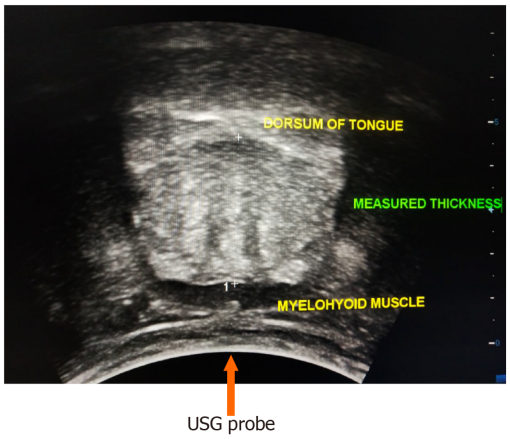


Figure 2 Vertical distance measured from the surface of the mylohyoid muscle to the tongue dorsum. USG: Ultrasonography.

data was carried out using the chi square test/Fisher's exact test. Continuous data were compared by the student *t*-test/Mann-Whitney test, as applicable. A *P* value less than 0.05 was considered significant. Intra-class correlation coefficient (ICC) was used to determine the reliability and agreement of the tongue thickness measurements.

RESULTS

Of 120 patients, 96 were males and 24 were females with a mean age of 47.12 years. The various aetiologies of cirrhosis were ethanol-related, non-alcoholic steato-hepatitis, cryptogenic, hepatitis B virus, hepatitis C virus, autoimmune, hepatic vein outflow tract obstruction, non-alcoholic fatty liver disease, and primary sclerosing cholangitis (Figure 3).

Tongue thickness and CTP score

Mean tongue thickness in patients with CTP class A was 4.39 ± 0.39 cm (range 4.25-4.53), in patients with CTP class B was 4.19 ± 0.53 cm (range 3.99-4.39), in patients with CTP class C was 3.87 ± 0.42 cm (range 3.71- 4.02) and in normal healthy individuals was 4.33 ± 0.49 cm (range 4.15-4.51) (Table 1; Figure 4).

A significant difference was seen in tongue thickness between patients with CTP class C and those with CTP class A and B ($P < 0.05$). Patients with CTP class C also had significantly reduced tongue thickness than normal individuals ($P < 0.05$). However, no significant difference was observed in tongue thickness between patients with CTP class A and B and normal individuals (Table 2).

Table 1 Tongue thickness in the study groups

Study group	mean ± SD	Median (range)
Child class A	4.39 ± 0.39 cm	4.50 cm (range 4.25-4.53)
Child class B	4.19 ± 0.53 cm	4.15 cm (range 3.99-4.39)
Child class C	3.87 ± 0.42 cm	3.80 cm (range 3.71-4.02)
Normal (healthy) subjects	4.33 ± 0.49 cm	4.35 cm (range 4.15-4.51)

SD: Standard deviation.

Table 2 Comparison of tongue thickness in the study groups

(I) CTP class	(J) CTP class	Mean difference (I-J)	Std. error	Significance	95%CI	
					Lower bound	Upper bound
Class A	Class B	0.2000	0.1186	0.335	−0.109	0.509
	Class C	0.5233[a]	0.1186	0.000	0.214	0.832
	Normal	0.0567	0.1186	0.964	−0.252	0.366
Class B	Class A	−0.2000	0.1186	0.335	−0.509	0.109
	Class C	0.3233[a]	0.1186	0.037	0.014	0.632
	Normal	−0.1433	0.1186	0.623	−0.452	0.166
Class C	Class A	−0.5233[a]	0.1186	0.000	−0.832	-0.214
	Class B	−0.3233[a]	0.1186	0.037	−0.632	-0.014
	Normal	−0.4667[a]	0.1186	0.001	−0.776	-0.158
Normal	Class A	−0.0567	0.1186	0.964	−0.366	0.252
	Class B	0.1433	0.1186	0.623	−0.166	0.452
	Class C	0.4667[a]	0.1186	0.001	0.158	0.776

[a]Statistically significant. CTP class: Child Turcotte Pugh class. CI: Confidence interval

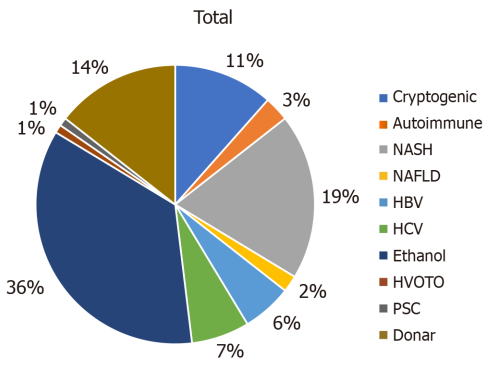


Figure 3 Aetiologies of liver cirrhosis.

MELD score

mean (± SD) MELD score in patients with CTP class A, B and C was 9.63 ± 2.24, 13.90 ± 2.96 and 25.37 ± 7.92, respectively.

Tongue thickness and MELD score

A statistically significant, negative correlation was found between MELD score and tongue thickness (r: −0.331) (P < 0.001) (Table 3).

Table 3 Correlation between "Model for end stage liver disease" score and tongue thickness

		Tongue thickness	MELD score
Tongue thickness	Pearson correlation	1	−0.330[a]
	Significance (2-tailed)		0.001
	n	120	90
MELD score	Pearson correlation	-0.330[a]	1
	Significance (2-tailed)	0.001	
	n	90	90

[a]Statistically significant. MELD score: Model for end stage liver disease score.

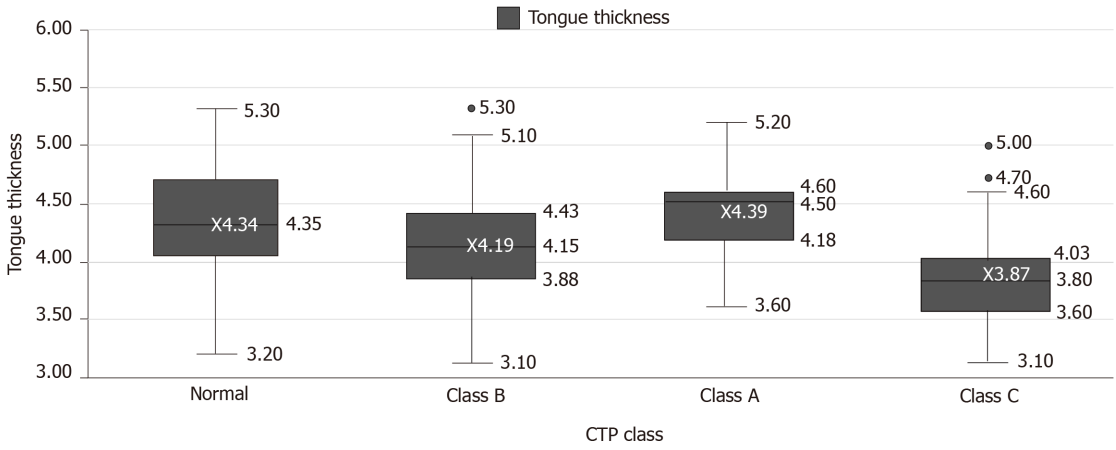


Figure 4 Box plot of the distribution of tongue thickness in different "Child Turcotte Pugh" class patients and normal healthy subjects.
CTP class: Child Turcotte Pugh class.

Age and tongue thickness

No significant correlation was found between age of the patients and tongue thickness by USG (Table 4).

Tongue thickness and L3SMI

No significant correlation was found between tongue thickness and L3SMI (P = 0.83) (Table 5). In healthy subjects, the mean (± SD) L3SMI value was 39.66 ± 6.8, and was 38.26 ± 8.88 in patients of CTP class C. The difference was not significant (P = 0.63) (Table 6). Barring the outliers, in 2 healthy subjects and in 2 CTP class C patients, the median L3SMI was 40.19 (33.89-45.4) and 39.39 (32.68-45.35), respectively (Figure 5). ICC was used to determine the reliability of tongue thickness measurements. The ICC value was 0.984 (95%CI: 0.979-0.989) and was indicative of good reliability (Table 7).

DISCUSSION

Our study indicates that tongue thickness measurement by USG correlates significantly with the severity of liver disease, as assessed by CTP scores. The study established that patients with a CTP score ≥ 10 have significantly reduced tongue thickness as compared to normal individuals and those with less severe liver disease with CTP scores of 5-9. Studies have shown that sarcopenia also affects other muscles besides the much studied L3SMI[7-9]. Tongue thickness has even been examined as a bedside measure of sarcopenia in patients with critical illness and has also been correlated with clinical outcome[9].

Malnutrition in cirrhosis is secondary to a multifactorial process and is seen more often in patients with more severe liver disease. We studied the correlation between tongue thickness and MELD score and found a significant negative correlation

Table 4 Correlation between age and tongue thickness

		Tongue thickness	Age
Tongue thickness	Pearson correlation	1	−0.081
	Significance (2-tailed)		0.382
	n	120	120
Age	Pearson correlation	−0.081	1
	Significance (2-tailed)	0.382	
	n	120	120

Table 5 Correlation between tongue thickness and L3 skeletal muscle index

		Tongue thickness	L3SMI
Tongue thickness	Pearson correlation	1	-0.074
	Significance (2-tailed)		0.424
	n	120	120
L3SMI	Pearson Correlation	-0.074	1
	Significance (2-tailed)	0.424	
	n	120	120

L3SMI: L3 skeletal muscle index.

Table 6 Comparison of L3 skeletal muscle index in Child Turcotte Pugh class C and normal healthy subjects

CTP class	L3SMI					*P* value
	Mean	Standard deviation	Median	Minimum	Maximum	
Class C	38.2613	8.88428	39.3900	18.37	52.85	0.63
Normal	39.6640	6.80565	40.1900	31.24	50.25	

L3SMI: L3 skeletal muscle index; CTP class: Child Turcotte Pugh class.

Table 7 Intraclass correlation coefficient for ultrasonography measurements of tongue thickness

	Intraclass correlation	95%CI		*F* test with true value 0			
		Lower bound	Upper bound	Value	df1	df2	Sig
Single measures	0.954[a]	0.939	0.966	63.487	119	238	0.000
Average measures	0.984[a]	0.979	0.989	63.487	119	238	0.000

[a]Statistically significant. Sig: Significance. CI: Confidence interval.

between the two (*r*: -0.331, $P < 0.01$), indicating that as the MELD score increased, tongue thickness decreased and may be interpreted as worsening of sarcopenia with worsening of liver disease. We also found a significant difference in tongue thickness between CTP class C patients and healthy individuals and between CTP class C patients compared to CTP class A and B patients. However, we did not find any significant difference in tongue thickness between healthy individuals and CTP class A and B patients. Apparently, an appreciable degree of sarcopenia manifests only later in the course of liver cirrhosis when a patient qualifies for CTP class C categorisation. Similar to our findings, Montano-Loza *et al*[11] studied 248 patients and found that sarcopenia was more prevalent in patients with CTP class C ($P < 0.05$) and in patients

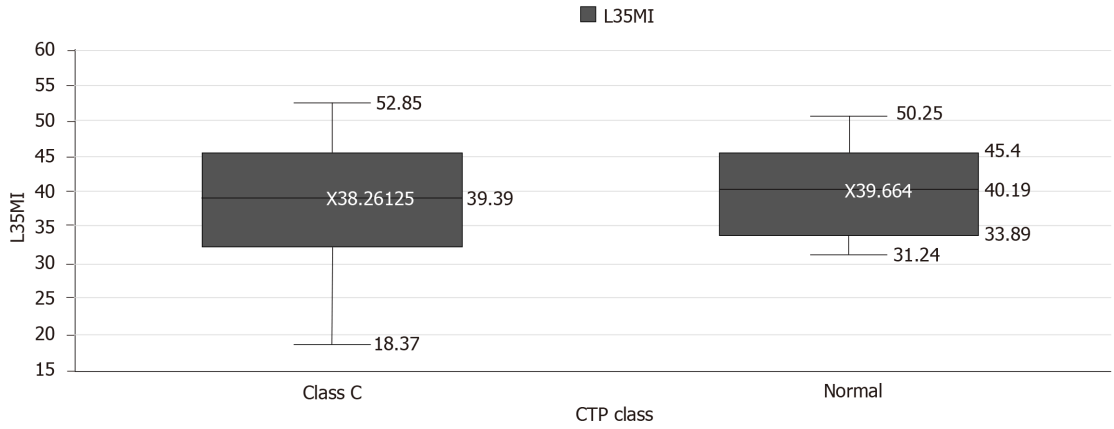


Figure 5 Box plot of the distribution of L3 skeletal muscle index in Child Turcotte Pugh class C patients and healthy individuals. L3SMI: L3 skeletal muscle index; CTP class: Child Turcotte Pugh class.

with higher MELD scores ($P < 0.02$). In other studies, Thandassery *et al*[6] and Tandon *et al*[12] also found a correlation between the prevalence of sarcopenia and disease severity, as measured by L3SMI and CTP.

However, in the present study, we did not find a correlation between tongue thickness and L3SMI. We also did not find any significant difference in L3SMI between healthy subjects and CTP class C patients. Measuring the mass of a muscle or group of muscles that predominantly have dynamic or postural functions, we believe, is flawed and the paravertebral muscles being postural muscles, are apparently only affected to an appreciable degree late in the disease course when patients may be critically ill and become bedridden. None of the patients included in this study were critically ill and/or admitted to the intensive care unit or were bedridden, and this could possibly be the reason why no difference was found between L3SMI in healthy subjects and CTP class C patients. This reasoning may also be inferred from the study of 116 patients with cirrhosis and hepatocellular carcinoma by Meza-Junco *et al*[13]. In their study, similar to our findings, the degree of sarcopenia measured using L3SMI did not correlate with CTP or MELD scores. However, in our study, tongue thickness was consistently and significantly decreased in CTP class C patients. Tongue thickness is probably a more sensitive marker of sarcopenia compared to L3SMI and requires further investigation.

A significant number of people more than 65 years old have decreased muscle mass[14]. In this study, we did not include patients more than 65 years, but we did not find a significant correlation between tongue thickness and age of the subjects included (Table 4). Sarcopenia in cirrhotic patients has been associated with increased mortality, sepsis, hyperammonemia, overt hepatic encephalopathy, and increased length of stay after liver transplantation[6]. The literature also suggests that patients with cirrhosis, poor nutritional status and sarcopenia have a higher risk of mortality, independent of the CTP and MELD scores[15]. It is therefore important to diagnose, quantify and perhaps classify the degree of sarcopenia for the medical management of patients with cirrhosis of the liver, for outcome prognosis and for planning interventions such as liver transplantation. This could be greatly helped by having a readily available and reproducible method for the diagnosis and quantification of sarcopenia.

The tongue has the advantages of direct inspection and ease of bedside measurement of thickness using USG, which is more readily available than CT scanning and is without the risk of radiation exposure, and unlike CT does not require extensive training. Tongue thickness measurement, as suggested by our findings, in addition to being objective, reproducible and easy, could be a more sensitive index for detecting sarcopenia than L3SMI. Our study was limited due to it being located at a single centre, and directed at patients with only a single disease, namely cirrhosis of the liver. Further studies exploring USG-measured tongue thickness in people of diverse ethnicity and different age groups in health and in disease, should be carried out to validate our findings and to establish this as a convenient bedside tool for diagnosing sarcopenia. In view of our study findings, we propose that tongue thickness measurement using USG should be considered for the diagnosis and quantification of sarcopenia.

ARTICLE HIGHLIGHTS

Research background

Sarcopenia in patients with chronic liver disease has prognostic implications. L3 skeletal muscle index (L3SMI) calculated from computed tomography (CT) images is currently the only objective and reproducible method accepted for the quantification of sarcopenia. This study aims to determine tongue thickness measured using ultrasonography as an alternative method for diagnosing sarcopenia.

Research motivation

Sarcopenia in patients with chronic liver disease has prognostic implications. Wider application of L3SMI calculated from CT images is limited by cost, the need for extensive training, limited availability and due to the risk of radiation exposure. Clinical researchers have suggested the inclusion of a measure of sarcopenia in established prognostic models for patients with liver disease. A dependable and reproducible method with wider availability is therefore needed.

Research objectives

This study aimed to examine tongue thickness measured using ultrasonography as a dependable bedside tool for the diagnosis of sarcopenia. Significant differences were seen in tongue thickness between healthy individuals and individuals with less severe liver disease compared to patients with more severe chronic liver disease.

Research methods

Patients with chronic liver disease and healthy individuals who satisfied the inclusion criteria underwent tongue thickness measurement using ultrasonography. The study was observational in nature and no intervention was planned on the basis of observations made. Tongue thickness measurements were compared between healthy individuals and patients with liver disease of different severity. The imaging technique used was ultrasonography, which has wider availability and does not involve radiation exposure unlike CT scanning used to measure L3SMI.

Research results

Significant differences were seen in tongue thickness between healthy subjects and patients with less severe liver disease compared to patients with more severe liver disease. Tongue thickness measured using ultrasonography is therefore proposed as a bedside measure of sarcopenia. However, its application requires further validation in studies involving subjects of different ethnicity, in health and in disease.

Research conclusions

This study established consistent and significantly reduced tongue thickness in patients with severe liver disease compared to healthy individuals and patients with less severe liver disease. Tongue thickness measured using ultrasonography may therefore be used as a bedside tool for the diagnosis of sarcopenia, an application with wide availability and no risk of radiation exposure compared to CT-based measurement of L3SMI.

Research perspectives

The findings in this study require validation in a similar study of tongue thickness using ultrasonography in people of different ethnicity in health and in disease.

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ORIGINAL ARTICLE

Retrospective Study

Role of telomere shortening in anticipation of inflammatory bowel disease

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Author contributions: Truta B designed the study, performed analysis and wrote the manuscript; Wohler E performed genetic analysis, interpreted the results, reviewed the manuscript; Sobreira N performed genetic analysis; Datta L contributed to data collection; Brant S contributed to study design, data interpretation and editing the manuscript.

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Abstract

BACKGROUND

The existence of genetic anticipation has been long disputed in inflammatory bowel disease (IBD) in the absence of the explanatory mechanism.

AIM

To determine whether it was predictive of genetic anticipation, we evaluated telomere length in IBD. We hypothesized that multiplex IBD families exhibit a genetic defect impacting telomere maintenance mechanisms.

METHODS

We studied three IBD families with multiple affected members in three successive generations. We determined telomere length (TL) in lymphocytes and granulocytes from peripheral blood of the affected members using flow cytometry and fluorescence in-situ hybridization (flow FISH). We also performed whole exome sequencing in the blood of all available family members and used PhenoDB to identify potential candidate gene variants with recessive or dominant modes of inheritance.

RESULTS

Out of twenty-four patients of European descent selected to participate in the study, eleven patients, eight parent-child pairs affected by IBD, were included in the genetic anticipation analysis. Median difference in age at diagnosis between two successive generations was 16.5 years, with earlier age at onset in the younger generations. In most of the affected members, the disease harbored similar

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gastrointestinal and extraintestinal involvement but was more aggressive among the younger generations. TL was not associated with earlier age at onset or more severe disease in members of successive generations affected by IBD. NOD2 gene mutations were present in the Crohn's disease patients of one family. However, no gene variants were identified as potential candidates for inheritance.

CONCLUSION
Telomere shortening appears unlikely to be involved in mechanisms of possible genetic anticipation in IBD. Further studies using a larger sample size are required to confirm or refute our findings.

Key words: Inflammatory bowel disease; Telomere; Anticipation; Genetic; Inheritance

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Core tip: This is a retrospective study to evaluate the role of telomere shortening in genetic anticipation of inflammatory bowel disease (IBD). Genetic anticipation is a long disputed concept in IBD and lacks an explanatory mechanism. We analyzed generational changes in telomere length of eight parent-child pairs of three generation IBD families with anticipation and performed whole exome sequencing to identify genetic variants for autosomal inheritance. Neither telomere shortening or autosomal inheritance was associated with anticipation in our three generation IBD families, suggesting other potential mechanisms underlie this phenomenon.

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INTRODUCTION

Genetic anticipation is a phenomenon in which an inherited disorder manifests at a younger age and often more severely in offspring than in the affected parent[1]. This phenomenon has been reported in over twenty monogenetic degenerative neurological disorders[2]. In some of these disorders, such as Huntington's disease, familial Parkinson's disease, and fragile X syndrome, the molecular basis of anticipation has been linked to the expansion of unstable genomic triplet repeats, in step with the progression of the disease down through generations[3-7].

For inflammatory bowel disease (IBD) the involvement of genetic anticipation has been considered since the 1990's[8,9] because of observed earlier ages at diagnosis and more severe disease among offspring of patients with IBD, mainly Crohn's disease (CD). However, no mechanisms accounting for genetic anticipation have been identified, and many scientists have doubted genetic anticipation in IBD, attributing the observed differences in age at diagnosis and disease severity to biased observation[10,11].

The list of conditions exhibiting anticipation continues to grow. This list includes not only monogenic disease but also polygenic diseases such as bipolar disorder[12], schizophrenia[13], rheumatoid arthritis[14], Behcet's disease[15], and chronic leukemia[16], in which the molecular mechanism is yet to be discovered.

One new cause of genetic anticipation is telomeropathies, which encompasses a wide variety of rare diseases caused by genetic defects in telomerase maintenance mechanisms[17,18]. Telomeres are nucleoproteins that protect chromosomal ends by counteracting the erosion caused by the problems inherent in terminal replication of the chromosomes. In telomeropathies, mutations in the telomerase reverse transcriptase, the specialized polymerase that synthesizes new telomere repeats, leads to progressive telomere shortening, causing earlier onset of disease in successive generations[18]. Families with telomere disorders, such as dyskeratosis congenita, Li-Fraumeni syndrome and Von-Hippel-Lindau disease display anticipation in generational changes, both in age at onset and in appearance of specific

symptoms[17-20]. Although genetic causes have been discovered for a set of identified telomere disorders, a number of related syndromes may likewise be due to unrecognized telomeropathies[21].

Interestingly, short telomeres were found to be associated with genetic anticipations in hereditary breast cancer (in women carrying a mutation in *BRCA1* or *BRCA2* genes, and a subset of *BRCAX* families)[22] and thyroid cancer[23]. There is increasing evidence suggesting that short telomeres and subsequent genomic instability contribute to malignant transformation[24,25]. Telomere shortening has been associated with dysplasia and neoplasia in the early onset of ulcerative colitis (UC)[26-28].

These findings generate thought-provoking questions about the possibility that telomere shortening may be the cause of genetic anticipation in other familial disorders associated with increased risk of malignancies such as IBD (which has an increased risk of cancer) - and a subset of these familial patients might result from unrecognized telomeropathies, particularly if there is also an accompanying identifiable genetic mutation related to telomere biology.

In our study, the primary objective was to evaluate generational changes in telomere length in parent-child pairs of familial IBD, to determine the role of telomere shortening, and its potential implication as a mechanism of anticipation in familial IBD. The secondary objective focused on investigating potential candidate gene variants for autosomal inheritance in these multi-generation IBD families.

MATERIALS AND METHODS

Study families

Study design: We selected three multi-generation IBD families included in the Johns Hopkins IBD Family Study. Each selected family had three or more first-degree relatives affected by IBD: Either CD, UC or IBD-unclassified (IBD-U). Affected and non-affected individuals completed a multiple-choice questionnaire. This questionnaire interrogated patient demographics, family history of IBD, household specifics to childhood such as water source, number of people in the house, birth order, passive and active smoking; questions also inquired about disease onset, disease location, extraintestinal manifestations and complications, as well as medical and surgical therapy for IBD. We selected those families where all three affected generations had access to similar therapy to avoid the confounding factor of different classes of therapy on severity of the disease. Therefore, most of the patients were treated with mesalamine, thiopurine and steroids. None of the patients were treated with methotrexate. The patients were treated before biologics were commonly used as part of the IBD therapy.

Blood samples also obtained from participants were used to isolate lymphocytes for storage, serum for serological analysis of antibodies and other proteins relevant to IBD and DNA for genotyping or sequencing.

The Johns Hopkins IBD Family Study, conducted with the overall goal of identifying IBD susceptibility genes, was initially approved in 1996 and later registered with clinical trials in 2010. The review of electronic and paper records and the genetic analysis for the current study was performed in 2019.

IBD diagnosis: To confirm the diagnosis of IBD, define the severity of the disease, and determine current and past therapies, we reviewed electronic and paper medical records including consult notes, imaging tests (computer tomography, upper gastrointestinal, small bowel and barium enemas studies), endoscopic (upper endoscopy, colonoscopy and capsule endoscopy) and pathology reports of all available family members. We used the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) IBD Genetic Consortium phenotype operating manual (version May 10, 2006) and forms to classify of disease as either CD, UC or IBD-U (https://repository.niddk.nih.gov/media/studies/ibd/ibd_phenotyping-manual.pdf).

Genetic analysis

Telomere length measurement: DNA was purified from whole blood using Qiagen Puregene kit. To measure average length of telomere repeats in cells, we used fluorescent *in situ* hybridization with labeled peptide nucleic acid probes specific for telomere repeats in combination with fluorescence measurements by flow cytometry (flow FISH) as previously described[29,30]. This method was developed to measure single-cell Telomere length (TL) using a fluorescently labeled probe that hybridizes to telomere DNA and has the unique advantage over other methods for measuring

telomere length by providing multi-parameter information on the length of telomere repeats in thousands of individual cells. The accuracy and reproducibility of the measurements are augmented by the automation of most pipetting (aspiration and dispensing) steps and by including an internal standard (control cells) with a known TL in every tube. Individuals were diagnosed with short telomeres if TL was shorter than the 10th percentile of the age-matched control population.

Whole exome sequencing: Performed on eight individuals from the three families as part of the Baylor-Hopkins Center for Mendelian Genomics. Whole exome sequencing (WES) was performed using Agilent SureSelect HumanAllExonV5Clinicalon the Illumina HiSeq2000 platform. Libraries were sequenced on the HiSeq2500 platform with onboard clustering using 125 bp-paired end runs and sequencing chemistry kits HiSeq PE Cluster Kit v4 and HiSeq SBS Kit v4. Fastq files were aligned with BWA mem[31] version 0.7.8 to the 1000 genomes phase 2 (GRCh37) human genome reference[32,33]. Duplicate molecules were flagged with Picard version 1.109(1716). Local realignment around indels and base call quality score recalibration were performed using the Genome Analysis Toolkit 26 version v3.1-1- g07a4bf8 or v3.3-0-g37228af. Variant filtering was done using the Variant Quality Score Recalibration (VQSR) method[34]. Variants that passed VQSR filtering were annotated using Annovar (version 2013_09_11) against a variety of data sources. Ultimately, PhenoDB[35] was used to analyze the data for coding, frameshifting, nonsense, or missense, rare (minor allele frequency > 1% in ExAC, gnomAD, 1000 Genomes, or Exome Variant Server) variants in genes that were shared between the three affected families based on recessive or dominant modes of inheritance.

Statistical analysis

Results are expressed as mean ± SD for n number of samples. Analysis of difference between groups was conducted using an analysis of variance, *t* test, or Fisher exact test, as appropriate. A two-sided *P* value < 0.05 was used for statistical significance. All analysis was done using GraphPad Prism software (GraphPad 5.0).

Approval of the study was obtained from the Institutional Review Board of Johns Hopkins University.

RESULTS

Clinical characteristics of patient population

There were twenty-four individuals selected to participate in the study. Only eleven out of the twenty-four were eligible (successive generations affected by IBD) for genetic anticipation analysis: Five patients with UC, four patients with CD and two patients with IBD-U. These represent eight parent-child pairs from the three families studied (Table 1).

IBD diagnosis confirmation: Through electronic and paper medical records review and the IBD phenotype was defined per NIDDK IBD Genetic Consortium phenotype operating manual (version May 10, 2006) to classify disease as either CD, UC or IBD-U.

Children's age at diagnosis: The median age of parents at diagnosis was 16.5 years older than that of the children affected by IBD and the median age at blood draw was 39.6 ± 23 years for the entire group. The mean age of parents at diagnosis was 40 ± 17 and the mean age of children was 21 ± 13, $P < 0.0014$. The UC families were of mixed descent Italian/Irish/Japanese and Poland/Russian/Puerto Rican while the CD family was of European descent (Polish/Russian). The later two families were also identified as being of Ashkenazi Jewish descent.

Disease extent and severity in parent-child pairs: Table 1 shows the extent of disease involvement in the CD, UC, and mixed families. Similar extent of the disease was noted in either parents or their children. In the family with CD, the disease extended into the large and small intestine in all three generations of affected individuals. All four affected members had a penetrating form (entero-enteral fistula, anal fistulas) of the disease. Arthropathy was the most common extraintestinal manifestation of IBD. In UC patients, the disease involved mainly the rectum and sigmoid colon (left side disease) and showed increasing severity as the disease was passed to the subsequent generation. All the affected members of the second family underwent total colectomy for disease refractory to medical therapy with the youngest member requiring surgery at only 6 years of age.

Table 1 Patient included in genetic anticipation analysis- characteristics and disease severity

ID	Member	IBD	Tobacco	AOD	NOD2	Extent	Behavior	Medications	Surgery
1-01	Mother	CD	None	21	+	Ileum, colon	B2	Steroids 5-ASA	Yes
1-02	Proband/F	CD	Former	9	+	Ileum, jejunum, colon	B3p	Steroids 5-ASA	No
1-05	Sister	CD	None	14	+	Ileum, colon	B2	Steroids 5-ASA thiopurine	No
1-07	Grandmother	CD	Passive	42	+	Ileum, colon	B2	5-ASA	Yes
2-01	Father	IBD-U	Active	21	_	Left colon	NA	5-ASA, thiopurine	Yes
2-02	Proband/F	UC	Active	4	_	Left colon	NA	Steroids 5-ASA, thiopurine	Yes
2-03	Grandfather	UC	Former	63	_	Left colon	NA	Steroids 5-ASA	Yes
3-01	Mother	UC	None	40	_	Left colon	NA	Steroids 5-ASA	Yes
3-03	Proband/M	UC	None	19	_	Left colon	NA	Steroids	No
3-04	Uncle	UC	Active	41	_	Left colon	NA	Steroids 5-ASA	No
3-05	Grandfather	IBD-U	Former	56	_	Pancolitis	NA	Steroids	No

There was no available TL data for the member 3-05. B2: Stricturing disease; and B3: Penetrating disease, as per Montreal Classification; Steroids: Refers to prednisone; Surgery: Refers to total colectomy for ulcerative colitis and segmental bowel resection for Crohn's disease. F: Female; M: Male; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Indeterminate colitis; 5-ASA: 5–aminosalicilates; AOD: Age at diagnosis; TL: Telomere length; NOD2: NOD2 gene; NA: Not applicable.

Results of genetic analysis

TL in successive generations: In order to evaluate the hypothesis that telomere shortening may be associated with the earlier onset of the disease, we investigated the mean relative TL of blood lymphocytes and granulocytes (Figure 1). Individuals were diagnosed with short telomeres if their telomere length was shorter than the 10th percentile of the age matched control population[17]. No associations were found between TL in lymphocytes and granulocytes and anticipation in the age at onset observed in successive generations.

WES for genetic inheritance: Finally, we explored the possibility of a gene variant with potential for autosomal inheritance in these Mendelian families. We used WES and PhenoDB to identify a genetic variant common to all three IBD families. NOD2 gene mutations were present in the CD affected patients of one family. WES did not detect any rare frameshift, nonsense or missense coding variants in genes shared by these three families for either dominant or recessive modes of inheritance. NOD2 gene mutations were present in the Crohn's affected patients of one family.

DISCUSSION

Awareness of the changing of disease pattern in IBD families with multiply affected generations not only has implications for genetic counseling and surveillance of at-risk members but also for implementation of correct treatment strategies. Genetic anticipation in IBD, although observed in most studies, has been questioned by many scientists due to lack of evidence for a molecular mechanism to explain this phenomenon. It has been suggested that anticipation may result from observation bias[10,11]. Recently, a Bayesian method (that corrects for random effects, isolating the confounding effect of changes in secular trends, screening and medical practices, and adjusts for changes in age-specific incidence across birth cohorts), confirmed anticipation observed among successive generations of Lynch Syndrome families, a syndrome that likewise had controversy regarding genetic anticipation[36]. Nevertheless, the molecular mechanism of genetic anticipation in Lynch Syndrome has not been identified. It is therefore possible, that genetic anticipation in IBD may be demonstrated using an optimal study design and statistical methods and/or evidence of a molecular mechanism[37].

In several monogenetic diseases, including Huntington disease[3], myotonic

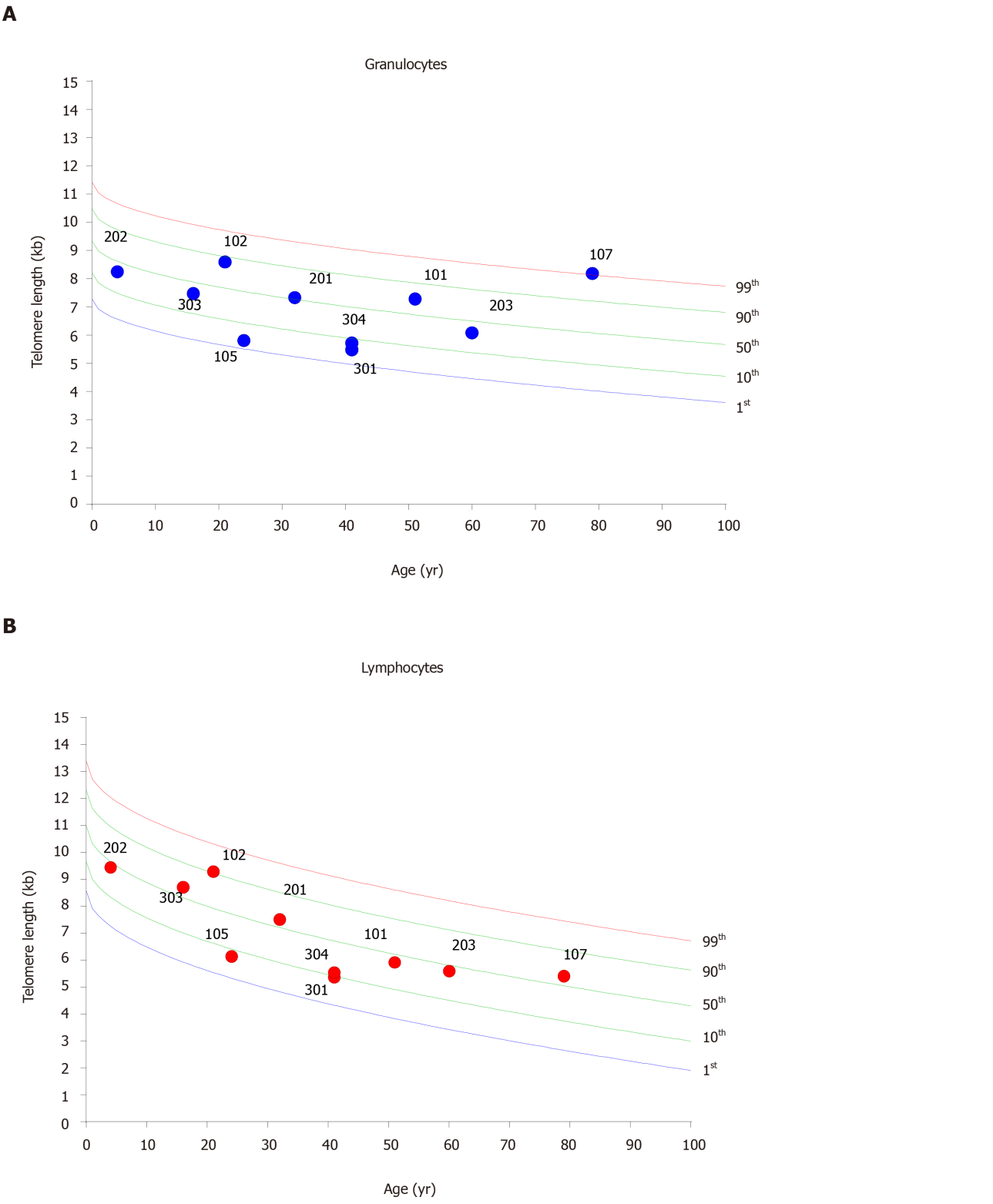


Figure 1 Telomeres length in the granulocytes and lymphocytes of affected members from the three inflammatory bowel disease families. Telomere length measured by flow cytometry with fluorescence in situ hybridization in affected individuals of Family 1, Family 2, and Family 3 plotted against nomogram from Johns Hopkins controls are within normal age adjusted range.

dystrophy[38], and Friedreich ataxia[39], the biological basis for genetic anticipation resides in expansion of unstable genomic triple repeats. In polygenic diseases, the complexity of multifactorial inheritance makes the identification of such molecular mechanism very challenging. In IBD, genomic imprinting of age at diagnosis has been proposed but when tested in 137 affected parent-child pairs from 96 families, it was not observed[40].

The patterns of genetic anticipation discovered for some telomeropathies generated

the question of whether a similar defect in telomere maintenance mechanisms may have a role in IBD. The TL has been studied in IBD in association with colon cancer and severity of inflammation in UC but not in the context of genetic anticipation[26-28,40]. In particular, we have questioned if these familial forms of IBD with observed anticipation might be due to unrecognized telomerophaties.

In our study, we examined three IBD families with clinical features of genetic anticipation. In all three families, the disease onset was reported at an early age (median 16.5) and became more severe in successive generations. In order to encompass three successive generations, we reviewed the disease history of families who received care at our center as early as 1996. Confusion related to the potential impact of newer therapy on the severity of the disease was avoided since all the recruited affected members had to receive treatment before the introduction of the biologics. Since population genetics has temporal stability, the recruitment methodology did not influence the results of the study which remain valid today despite the age of our families.

We have shown that when controlling for age, no association exists between TL and age at disease onset among members of successive generations affected by IBD. Genetic anticipation is unlikely to be the result of telomere shortening and other mechanism should be explored. We also investigated the potential for these IBD families with Mendelian pattern of inheritance to represent a monogenic form of IBD similar to Very Early Onset IBD[41]. In particular, we wanted to learn if there is an accompanying identifiable genetic mutation related to telomere biology[32] in these families and if its effects were overlooked due to our study sample size. Moreover, in some monogenic diseases, genetic anticipation has been linked to the expansion of unstable genomic triplet repeats[3], a mechanism that we would have explored in IBD had it been monogenetic. However, besides the *NOD2* mutations identified among the affected members one of the CD families, WES did not detect any rare frameshift, nonsense or missense coding variants in genes shared by these three families for either dominant or recessive modes of inheritance. In adults, *NOD2* is typically the most highly penetrant established gene associated with CD but the population attributable risk of *NOD2* variance to CD is only 26%[43]. No mutation in genes involved in the telomere maintenance mechanism were detected.

Strengths and limitations

The study represents the first evaluation of TL defects in genetic anticipation of IBD. Even though no association was found between TL and age at disease onset among members of successive generations, this pilot study is very valuable as an attempt to find the molecular mechanism of genetic anticipation in IBD. While we have shown that genetic anticipation is unlikely to be the result of telomere shortening, our results do point the way to explore other mechanisms. Nonetheless, the sample size may be too small to rule out whether telomeropathies may play a role in other families with similar strong evidence for anticipation. Further studies to confirm our findings should address a larger population of familial IBD that likewise have strong evidence for anticipation among successive generations.

ARTICLE HIGHLIGHTS

Research background

The existence of genetic anticipation, a phenomenon in which an inherited disorder manifests at a younger age and often more severely in offspring than in the affected parent, has been long disputed in inflammatory bowel disease (IBD). In the absence of the explanatory mechanism, it has been suggested that anticipation may result from observation bias. In monogenetic degenerative neurological disorders the mechanism for genetic anticipation was identified as unstable genomic triple repeats. In polygenic diseases, such as IBD, the complexity of multifactorial inheritance makes the identification of genetic mechanism very challenging. I Most recently, mutations in the telomerase reverse transcriptase, have been shown to lead to progressive telomere shortening, causing earlier onset of disease in successive generations with telemeropahities. Telomere length (TL) has also been found to be associated with severity of inflammation and colon cancer in IBD.

Research motivation

The role of TL in genetic anticipation of patients with IBD has not been studied.

Research objectives

In our study, the primary objective was to evaluate generational changes in TL in parent-child pairs of familial IBD, to determine the role of telomere shortening, and its potential implication as a mechanism of anticipation in familial IBD. The secondary objective focused on investigating potential candidate gene variants for autosomal inheritance in these multi-generation IBD families.

Research methods

We studied three IBD families with multiple affected members in three successive generations. We determined TL in lymphocytes and granulocytes from peripheral blood of the affected members using flow cytometry and fluorescence in-situ hybridization (flow FISH). We also performed whole exome sequencing (WES) in the blood of all available family members and used PhenoDB to identify potential candidate gene variants for recessive or dominant modes of inheritance.

Research results

Out of twenty-four patients of European descent selected to participate in the study, eleven patients, eight parent-child pairs affected by IBD, were included in the genetic anticipation analysis. Median difference in age at diagnosis between two successive generations was 16.5 years, with earlier age at onset in the younger generations. Five patients were affected by ulcerative colitis, four patients by Crohn's disease and two patients by IBD-Unclassified. In most of the affected members, the disease harbored similar gastrointestinal and extraintestinal involvement but was more aggressive among the younger generations. TL was not associated with earlier age at onset or with more severe disease in members of successive generations affected by IBD. NOD2 gene mutations were present in the Crohn's affected patients of one family. However, no gene variants were identified as potential candidates for Mendelian inheritance.

Research conclusions

The study represents the first evaluation of TL defects in genetic anticipation of IBD. Even though no association was found between TL and age at disease onset among members of successive generations, this pilot study is very valuable as an attempt to find the molecular mechanism of genetic anticipation in IBD.

Research perspectives

While we have shown that genetic anticipation is unlikely to be the result of telomere shortening, our results do point the way to exploration of other mechanisms. Further studies to confirm our findings should address a larger population of familial IBD that likewise have strong evidence for anticipation among successive generations.

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ABOUT COVER

Editorial board member of *World Journal of Gastrointestinal Pharmacology and Therapeutics*, Dr. Amir Abbasnezhad is a Distinguished Professor at the Lorestan University of Medical Sciences, Iran. Dr. Abbasnezhad completed his PhD in Nutrition at Ahvaz Jundishapoor University of Medical Sciences and his undergraduate studies at Urmia University. His research interests lie in the area of nutrition, gastroenterology, and complementary medicine; in addition, he has actively collaborated with researchers in several other medical disciplines, particularly in the fields of cancer and gastrointestinal disorders, such as inflammatory bowel diseases. In 2016, he became Deputy of Research of the Nutritional Health Research Center of the Lorestan University of Medical Sciences and won the Specialized Duty System Award from the Iran's National Elites Foundation. In 2017, 2018 and 2019, he consecutively won the Top Researcher Award from the Lorestan University of Medical Sciences. (L-Editor: Filipodia)

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ORIGINAL ARTICLE

Basic Study

Oral encapsulated transforming growth factor β1 reduces endogenous levels: Effect on inflammatory bowel disease

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Author contributions: Auci DL and Hammer L designed and coordinated the studies, analyzed data and wrote the manuscript; Hammer L performed in life phases and serum and tissue analysis; Furtado S, and Mathiowitz E prepared drug products and reviewed the manuscript.

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Conflict-of-interest statement: Dr. Auci reports grants from NIH, during the conduct of the study; In addition, Dr. Auci has a patent

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Abstract

BACKGROUND

TreXTAM® is a combination of the key regulatory cytokine transforming growth factor beta (TGFβ) and all trans retinoic acid (ATRA) microencapsulated for oral delivery to immune structures of the gut. It is in development as a novel treatment for inflammatory bowel disease (IBD).

AIM

To measure TGFβ levels in blood and tissue after oral administration of encapsulated TGFβ.

METHODS

Animals were orally administered encapsulated TGFβ by gavage. Levels of drug substance in blood and in gut tissues at various times after administration were measured by ELISA.

RESULTS

We made the surprising discovery that oral administration of TreXTAM dramatically (approximately 50%) and significantly (P = 0.025) reduced TGFβ levels in colon, but not small intestine or mesenteric lymph nodes. Similarly, levels in rat serum after 25 d of thrice weekly dosing with either TreXTAM, or microencapsulated TGFβ alone (denoted as TPX6001) were significantly (P < 0.01) reduced from baseline levels. When tested in the SCID mouse CD4+CD25- adoptive cell transfer (ACT) model of IBD, oral TPX6001 alone provided only a transient benefit in terms of reduced weight loss.

CONCLUSION

These observations suggest a negative feedback mechanism in the gut whereby

Micronized freeze-dried particles issued, and a patent Compositions for stabilizing and delivering proteins pending and Authors hold equity in Therapyx.

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local delivery of TGFβ results in reduced local and systemic levels of the active form of TGFβ. Our findings suggest potential clinical implications for use of encapsulated TGFβ, perhaps in the context of IBD and/or other instances of fibrosis and/or pathological TGFβ signaling.

Key Words: Transforming growth factor beta; All trans retinoic acid; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease; Regulatory T cells

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Core Tip: The observations suggest a negative feedback mechanism in the gut whereby local delivery of transforming growth factor beta (TGFβ) to immune structures of the gut results in reduced local and systemic levels of the active form of TGFβ.

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INTRODUCTION

TreXTAM® is a proprietary micro-encapsulated drug product in development as an oral treatment for inflammatory bowel disease (IBD). It is the combination of the key regulatory cytokine transforming growth factor beta (TGFβ) encapsulated in into poly-lactic acid (PLA) particles; along with a signaling form of vitamin A, all trans retinoic acid (ATRA), encapsulated in poly D,L-lactide-co-glycolide (PLGA) particles[1]. Simultaneous ATRA and TGFβ signals synergize in promoting the differentiation and stabilization of regulatory T cells[2]. This is a completely novel strategy for the treatment of IBD, as no similar products exist. However, unlike ATRA, TGFβ is a protein macromolecule that must be protected against hydrolysis in the stomach to be effective *via* the oral route[3].

Encapsulation remains one of the most promising methods to protect drug substances and to achieve local, sustained release. Efforts have generally focused on siRNA[4], small molecules[5], peptides[6] and cytokines[7] and involve polymer encapsulation accomplished *via* combinations of phase separation or precipitation, emulsion/solvent evaporation[8-15] and/or spraying methods[16-20]. However, loss of bioactivity during manufacturing, poorly controlled release rates, and difficulties with large-scale production of accurately sized particles are some of the formidable challenges preventing commercialization.

To address these challenges, we pioneered the development of phase inversion nano-encapsulation (PIN®) technology that utilizes a non-mechanical approach to preserve the structural integrity of macromolecules during the drug product manufacturing process. PIN encapsulated cytokines have demonstrated stability, bioactivity and efficacy in various preclinical models[21-26]. Particles with an average diameter of 0.1-5 microns[6], are ideally suited to oral delivery as particles smaller than 5 microns in diameter readily traverse the gastrointestinal barrier[27-29]. Indeed, we had previously shown that orally administered insulin encapsulated in PIN particles resulted in localization of drug product to the gut, and efficient uptake at the intestinal border[6,7].

More recently we applied PIN technology to the development of TreXTAM and showed that oral administration effectively ameliorated disease in two different rodent IBD models[1]. Broadly, treatment of mice with established disease using the optimized dose/frequency regimen, achieved a dramatic 2 to 9-fold reduction in multiple markers of disease compared to control groups within 2 wk, in some cases approaching normal values. Importantly, treatment enhanced long-term survival over eight weeks with no detectable toxicity. Activity was associated with enhanced Foxp3 expression in the colonic lamina propria CD4+ CD25+ T-cells, and required both TGFβ and ATRA for maximal efficacy. We have recently reviewed potential cellular and

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molecular mechanisms driving synergy, including cross-talk between ATRA and TGFβ signal transduction pathways[2].

During TreXTAM development, we studied TGFβ pharmacokinetics after oral administration of TreXTAM, or after the encapsulated cytokine (TPX6001) was given alone, without ATRA. We made the surprising discovery that oral administration of TreXTAM dramatically reduced TGFβ levels in colon and in blood, to below baseline levels. When encapsulated TGFβ (TPX6001) was given alone, three times a week for 25 d, we likewise observed serum TGFβ decreases below baseline (untreated) levels. Oral treatment with TPX6001 alone transiently ameliorated weight loss in the murine adoptive cell transfer (ACT) model of IBD. These observations suggest a negative feedback mechanism in the gut whereby local delivery of TGFβ results in reduced local and systemic levels of the active form of TGFβ. This finding suggests potential clinical implications for use of encapsulated TGFβ in the context of IBD and/or pathological TGFβ signaling.

MATERIALS AND METHODS

Preparation and characterization of TGFβ and ATRA loaded formulations

Microsphere preparation: For tissue studies, TGFβ (Peprotech, Rocky Hill, NJ, United States) was encapsulated into bench-top scale, poly-lactic acid (PLA) particles (0.285 mg TGFβ per gram of final drug product; for simplicity and clarity the abbreviation TGFβ refers specifically to TGFβ1, unless otherwise noted) using PIN as described previously[30]. ATRA (Sigma) was encapsulated into poly-lactic-co-glycolic acid (PLGA) particles (1 mg of ATRA per gram of particles) using a modification of the solvent evaporation technique as in previous studies[31].

For PK studies, TGFβ and ATRA loaded microspheres (denoted TPX6001 and TPX7001, respectively) were synthesized at Lonza-Bend, Bend Oregon using a proprietary two-step spray dry process to manufacture larger, scaled up quantities. Briefly, in Step 1, lyophilized protein is mixed with excipients and dispersed. In Step 2, micronized protein + excipients are encapsulated, precipitated and collected. To reduce dose mass, TGFβ and ATRA spray dry drug products were loaded at 1 mg/g and 2 mg/g w/v. The release kinetics, bioactivity, morphology, long-term (1 year) stability, as well as the physicochemical properties of glass transition temperature and crystallinity were essentially identical in the bench lots and spray dried particles (data not shown).

TGFβ and ATRA loaded PLA and PLGA particles were mixed cage-side in the indicated proportions to create TreXTAM, a proprietary combinatorial product designed to provide both TGFβ and ATRA signals thought to drive the development of regulatory T cells[32-35].

***In vitro* drug substance release:** Formulations were release-tested using an *in vitro* release assay described previously[24]. Briefly, for TGFβ, 0.2 mL of a 10 mg/mL particle suspension was transferred to the wells of a 96-well plate in triplicate. The plate was incubated at 37 °C in 5% CO_2, the supernatants were sampled at the indicated time points and stored at -20 °C until use. ATRA was extracted and measured by HPLC as in our previous studies[36]. The immune-reactive, active form of TGFβ was measured by assaying non-acidified samples in an ELISA (R&D Systems Quantikine ELISA kit Catalog# MB100B). This assay does not have significant cross-reactivity or interference with TGFβ2 or TGFβ3, and does not detect the latent form of TGFβ1 without acid treatment. ATRA extraction and analysis was performed as follows: 10 ± 0.1 mg ATRA containing microspheres were weighed into 15 mL Falcon tubes for each terminal time-point. 1 mL of 1 × PBS was added and placed on end-over-end rotator at 37 °C. At predetermined time-points, the tubes were centrifuged and supernatant discarded. The remaining microspheres were flash frozen and lyophilized for 24 h. Microsphere samples were then extracted by adding 5 mL of pH 7 mobile phase (68:24:8 ratio of acetonitrile: 1% glacial acetic acid:ethanol) and bath sonicating for 45 min. Extracted samples were then run on a HPLC using a Waters Symmetry C18 Column (5.0 μm, 3.9 mm × 150 mm) at a flow rate of 1 mL/min using pH 7 mobile phase. Absorbance was measured at 356 nm.

Pharmacokinetic studies

Animals: The in-life phase of these studies was performed at Comparative Biosciences, Sunnyvale, California. 7 to 9-wk old Sprague-Dawley rats (males and females) were kept under standard laboratory conditions with free access to food and water. They

were allowed to adapt one week before starting the study. The care and use of laboratory animals was in accordance with relevant IACUC-approved animal use protocols.

Administration of encapsulated drug products: A 0.5-mL aliquot of TreXTAM (or TPX6001 alone) in aqueous suspension was prepared by reconstitution of drug products (TPX7001 and/or TPX6001) with distilled water and mixed in appropriate w/v proportions to achieve the targeted dosing. Animals were dosed by oral gavage. Blood samples were collected at fixed times after dosing.

Tissues analysis: 7 to 9-wk old male Sprague-Dawley rats n = 3 per group) were untreated, or treated (oral gavage) with TreXTAM three times per week for four weeks. Four hours after the final dose, gut tissues were taken, frozen at -20 °C and stored until used. Tissues were then thawed and homogenized using a glass tube with the pestle insert, in the presence of EDTA-free SIGMAFAST™ Protease Inhibitor Cocktail Tablets (Sigma-Aldrich) used as per manufacturer's instructions. Levels of TGFβ1 and ATRA in lysates were measured as described above.

Serum analysis: Serum levels of TGFβ1 were measured using an ELISA kit (R&D Systems, Minneapolis, MN; see above) with a slight modification from manufacturer's instructions. Samples were not acid- activated, minimizing detection of endogenous latent cytokine. For ATRA, a high-performance liquid chromatograph combined with a triple quadrupole mass spectrometer was used as in our previous studies[36].

SCID mouse CD4+CD25- T cell transfer colitis model

The model was chosen because it recapitulates a regulatory T cell immunological basis of colitis, and was performed as in our previous studies[1]. Briefly:

Animals: Six to 8-wk old BALB/c and CB-17 SCID mice (males and females; Jackson Laboratories, Bar Harbor, MA, United States) were kept under standard laboratory conditions with free access to food and water and allowed to adapt one week before starting the study. The care and use of laboratory animals was in accordance with a University at Buffalo IACUC-approved animal use protocol.

Isolation of CD4+CD25- T cells: CD4+ CD25- T-cells were purified from the spleens of naïve BALB/c mice by magnetic bead separation using MACS® column and separator according to manufacturer's instructions (Miltenyi Biotech, San Diego, CA, United States). Purity and viability (> 95%) were assessed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, United States).

Induction of colitis: Purified CD4+CD25- T-cells were adoptively-transferred to SCID recipients (4×10^5 cells per mouse, i.p.). Mice were randomized into groups when 10% of mice show 5% or greater weight loss and/or soft or bloody stools and treatment (3 × per week *via* oral gavage) started. Daily disease score was recorded for each animal as in our previous studies[1] and summarized for each group as cumulative disease score during treatment. Last recorded values of animals that died during treatment were brought forward. At the end of the treatment period, all mice were sacrificed, and colons scored grossly for pathology on a 0 (normal) to 5 (diseased; elongated, inflamed, lacking definable stools) scale. Histology was also performed as in our previous studies[1]. Six to eight H&E sections of colon representing ascending, transverse and descending colon per mouse were evaluated independently, in blinded fashion, by a board-certified pathologist (Pacific Tox Path, LLC, Ellensburg, WA, United States). A composite inflammation score was calculated based on (0-3) severity and extent of cellular infiltration, amount of mucus and degree of proliferation (maximum score of 12).

Statistical analysis

Significance ($P \leq 0.05$) between experimental and control groups was determined using Student's t-test analysis. In experiments with multiple groups, homogeneity of inter-group variance was analyzed by ANOVA.

RESULTS

In vitro release patterns of TGFβ and ATRA and typical appearance of PLA and PLGA microsphere particles

For tissue studies, and for ACT studies, TGFβ and ATRA were encapsulated using PIN or solvent evaporation techniques bench top-processes (respectively) at 0.285 mg/g and 1 mg/g w/w, respectively. At 24 h, both TGFβ and ATRA drug products released bioactive (confirmed using TGFβ sensitive mouse lymphoblast cell line HT-2 or ATRA sensitive murine melanoma B16-F1cells; data not shown) TGFβ or ATRA (Figure 1A and B respectively) as expected, indicating that both drug substances could potentially be delivered in active forms, simultaneously, *in vivo* after oral administration.

TGFβ and ATRA in small and large intestine and MLN after oral Administration of TreXTAM to male rats

To assess delivery of ATRA and TGFβ to gut, male Sprague-Dawley rats were fed with either blank particles or with TreXTAM (60 mg/kg and 30 mg/kg of TGFβ and ATRA loaded particles; denoted TPX6001 and TPX7001, respectively, and loaded at 0.286 mg/g and 1 mg/g, approximately 17 and 30 μg/kg respectively) three times per week for four weeks. Four hours after the final treatment, small intestine, large intestine and MLN were collected from each animal and frozen at -20 °C. ATRA and TGFβ levels in small and large intestine, as well as MLN, were determined by HPLC or ELISA, respectively (limit of detection 0.75 ng/mL and 0.4 pg/TGFβ/100 μg of protein, respectively). Levels of ATRA in small intestine and MLN of treated and untreated animals were at the limit of detection. Levels of ATRA in colon were virtually the same in treated and untreated animals (data not shown). TGFβ was also negligible in small intestine and MLN of treated and untreated animals. However, TGFβ levels in colon of treated animals were decreased over 50% compared to untreated animals (Figure 2). This difference was significant (P = 0.025) suggesting a treatment associated attenuation of endogenous active TGFβ in colon tissue. Since those initial studies, we scaled up production of PLA encapsulated TGFβ (TPX6001) using the proprietary two-step stray dried manufacturing process described in the methods section. Production of PLGA encapsulated ATRA (TPX7001) has also been scaled up using spray drying methods. Release rates and physiochemical properties of the spray dried and bench top materials were virtually identical (data not shown). All pharmacokinetic work to follow was performed using spray-dried TGFβ PLA and ATRA PLGA (loaded at 0.1 and 0.2%, respectively) material.

Pharmacokinetics following oral administration of TreXTAM

We could not directly demonstrate simultaneous delivery of TGFβ or ATRA to gut tissue by oral TreXTAM (although we could see biological effects[1]). To further investigate this issue *in vivo*, and as part of our development efforts, we tested oral TreXTAM in a 28-d GLP rat toxicology study. The relevant pharmacokinetic for ATRA after TreXTAM administration has been published previously[36]. Those studies reported that after a single oral TreXAM administration, serum ATRA levels peaked with a T_{max} of 60 min and t ½ of 143 min.

We report here that after oral administration of TreXTAM (30 mg/kg spray-dried encapsulated TGFβ and 30 mg/kg PLGA encapsulated ATRA) three times a week for 25 d, serum TGFβ levels were significantly reduced compared to those observed in the same animals on day 0, prior to any TreXTAM dosing (Figure 3; NB: The level of ELISA detection is approximately 150 pg/mL). This finding was reminiscent of our observations of reduced TGFβ in colon after dosing (Figure 2). We also note that in the pre- dose, naïve animals n = 24 per sex) females had higher endogenous levels of TGFβ compared to males (492 ± 107 pg/mL *vs* 324 ± 14 pg/mL). This difference was highly significant (P < 0.0001). Similar observations were reported previously by Knabbe *et al*[37].

Oral treatments with PLA encapsulated TGFβ reduce serum levels of TGFβ

We also tested spray-dried PLA encapsulated TGFβ (TPX6001; loaded at 1 mg/g w/w) given alone in a similar 28-d GLP rat toxicology study (Figure 4). Once again, when similar analyses were performed on naive animals and on the same animals that had been dosed three times per week for 25 d, a dramatic and highly significant (P < 0.01) treatment-related reduction in serum TGFβ levels was evident for all dose groups (Figure 4). Indeed, the reduction in baseline serum TGFβ was dose dependent, in that the difference between the low and high dose group was also significant (P < 0.03). It

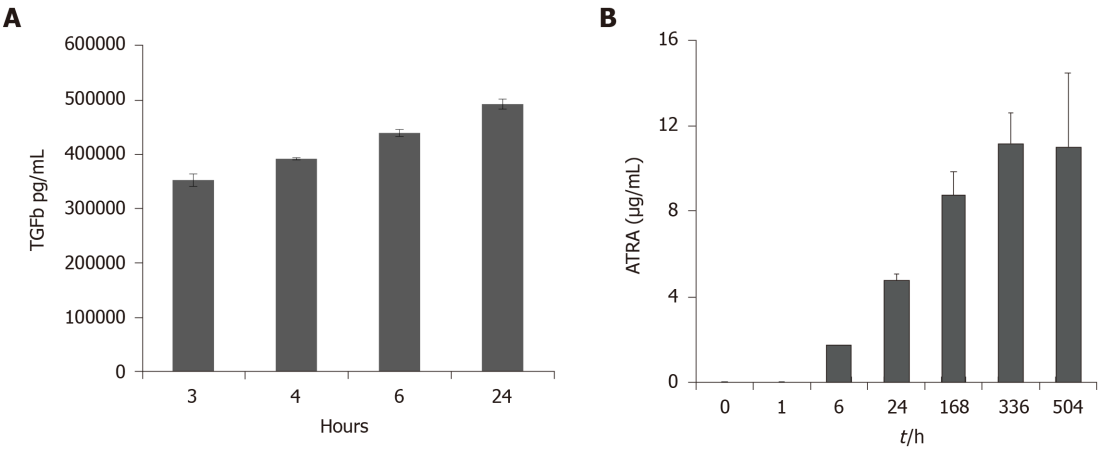


Figure 1 Release profiles of transforming growth factor -loaded poly-lactic acid microspheres and all trans retinoic acid-loaded poly-lactic-co-glycolic acid microspheres. Transforming growth factor (TGF) was encapsulated in poly-lactic acid (PLA) microspheres (285 μg of TGF per gram of particles) using Phase Inversion Nano-encapsulation (PIN). All trans retinoic acid (ATRA) was encapsulated into poly-lactic-co-glycolic acid (PLGA) microspheres (1 mg of ATRA per gram of particles) using a modification of the solvent evaporation technique (see methods section). A: TGF-loaded microspheres were release-tested using the *in vitro* release assay as described in the methods section; B: ATRA-loaded microspheres were release-tested using an *in vitro* extraction assay as described in the methods section. Data are expressed as pg/mL or as μg/mL ± SE. TGFβ: Transforming growth factor ; ATRA: All trans retinoic acid.

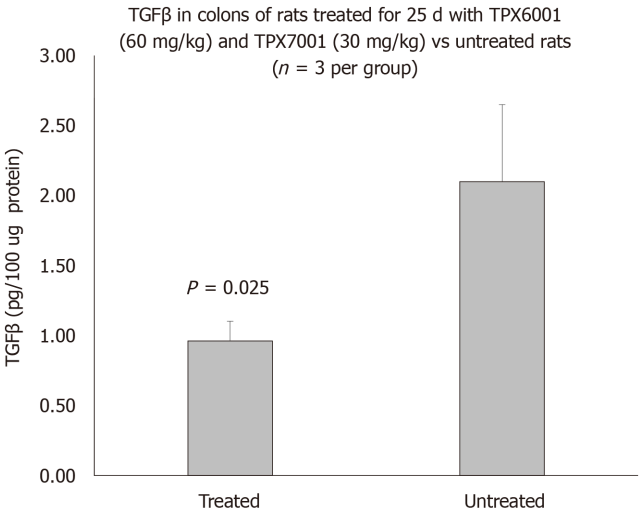


Figure 2 Effect of oral treatment with TreXTAM on levels of transforming growth factor in colon. Treated rats (*n* =3) received TreXTAM [60 mg/kg encapsulated transforming growth factor (TGF) and 30 mg/kg encapsulated all trans retinoic acid (ATRA)] three times a week for four weeks. Colons of these animals were taken 4 h after the final dose, along with tissues from age and sex matched untreated animals. All tissues were frozen at -20 °C and stored until used. Tissues were then thawed and homogenized using a glass tube with the pestle insert, in the presence of EDTA-free SIGMAFAST™ Protease Inhibitor Cocktail Tablets (used as per manufacturer's instructions). Levels of TGF in lysates were determined by ELISA according to manufacturer's instructions but without acid activation. (Quantikine, R&D Systems, Minneapolis, MN, United States). Total protein concentration was determined by BCA Protein Assay- Pierce (Thermo Fisher Cat# 23227) Data are expressed as pg/100 μg protein ± standard deviation. TGFβ: Transforming growth factor .

was also interesting to once again note that in naïve pre-dose animals n = 24 per sex), females had significantly (P = 0.001) higher levels of TGFβ than males, (492 ± 107 pg/mL *vs* 324 ± 14 pg/mL) indicating the same gender bias.

Effect of TPX6001 on disease in the SCID mouse CD4+CD25- ACT model of IBD

We next tested the IBD therapeutic potential of TPX6001 oral treatments when given alone, without ATRA, in the SCID mouse ACT model of IBD. This single, preliminary study used a highly challenging therapeutic iteration of the model. Treatments began at disease onset. There were no significant differences between groups in terms of body weight or disease score at the start of treatment. We found that TPX6001 treatment resulted in significant attenuation of weight loss (Figure 5). The differences between the 5 mg and 40 mg doses (days 3 to 12) were significant (P = 0. 01) compared to animals treated with blank microspheres. The difference between the 10 mg and blank groups during that same period achieved only a trend (P = 0.12), possibly

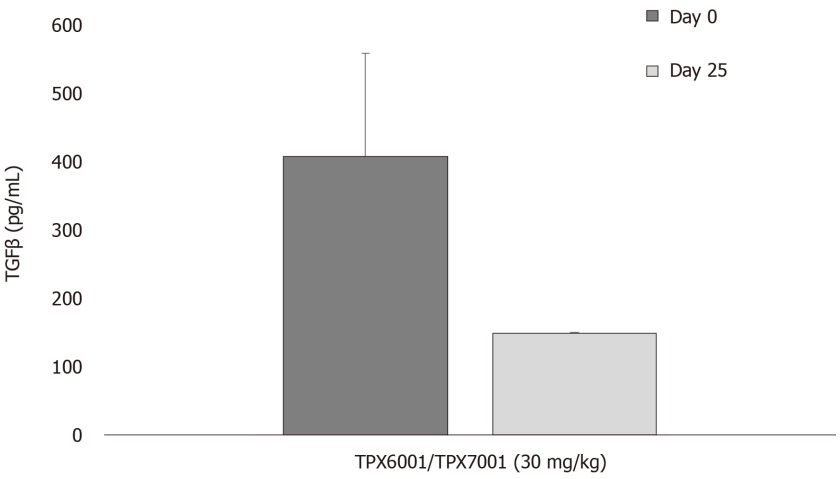


Figure 3 Baseline serum levels of transforming growth factor in naïve and TreXTAM treated animals. Blood was taken from naïve male and female Sprague-Dawley rats (males and females, 3 per sex) before and after treatment with TreXTAM [30 mg/kg encapsulated transforming growth factor (TGF) and 30 mg/kg encapsulated all trans retinoic acid (ATRA)]. Animals were dosed by gavage 3 × per week for 25 d. Serum levels of TGFβ were determined by ELISA without acid activation (R&D Systems Quantikine ELISA Catalog# MB100B). TGFβ: Transforming growth factor .

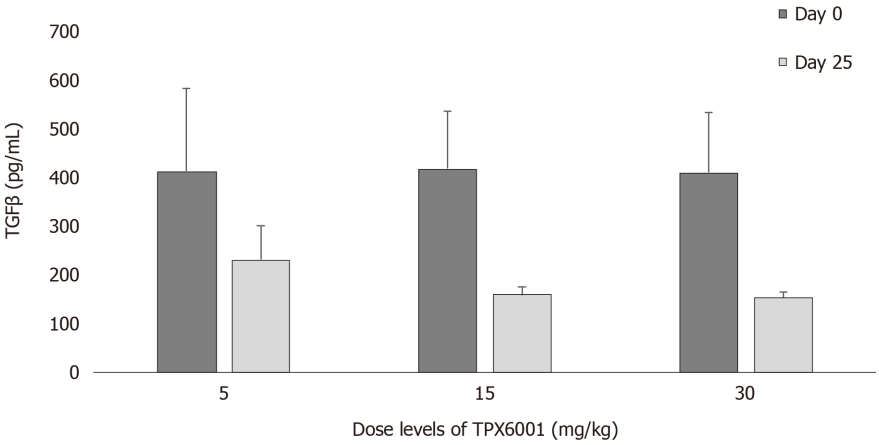


Figure 4 Baseline serum levels of transforming growth factor in naïve rats and in poly-lactic acid encapsulated transforming growth factor treated rats. Blood was taken from naïve Sprague-Dawley rats (males and females, 6 per sex) before and after treatment with poly-lactic acid encapsulated transforming growth factor (TGF). Animals were treated by gavage at doses of 5, 15 or 30 mg/kg, 3 × per week for 25 d. Serum levels of TGFβ were determined by ELISA without acid activation (R&D Systems Quantikine ELISA Catalog# MB100B). Statistical significance ($P \leq 0.02$) *vs* day 25 at 5 mg/kg *vs* day 25 at 30 mg/kg. All differences between day 0 pre-dose and day 25 pre-dose were significant ($P \leq 0.01$). TGFβ: Transforming growth factor .

because of two deaths in the 10 mg group. It is also interesting to note that the high dose group showed the most benefit for the first 7 d of treatment, but then deteriorated rapidly. At the end of the study, for each group, we calculated cumulative disease score during treatment (blank fed group = 52; 5, 10 and 40 mg treatment groups = 49.5, 52.6, and 47, respectively); colon weight to length ratios (blank fed group = 55.3 ± 14.3; healthy age and sex matched controls = 27.9 ± 4.4; 5, 10 and 40 mg treatment groups = 52.8 ± 9.1, 56.5. ± 15.3, and 57.4 ± 9.9, respectively), gross pathology (blank fed group = 2.9 ± 0.9; 5, 10 and 40 mg treatment groups = 3.7 ± 1.4, 2.4. ± 1.4, and 3.8 ± 1.1, respectively) and histology composite inflammation scores (blank fed group = 8.75, and 5, 10 and 40 mg treatment groups = 9.4, 7.8, and 8.1, respectively). We found no significant differences, except at the 10 mg dose ($P = 0.003$), which again, may have been biased by the deaths of 2 animals in that group. We also note a trend in favor of treatment with respect to cumulative disease score, in the high dose group ($P = 0.08$). Therefore, we conclude only slight, transient benefit of TPX6001 treatment in this iteration of the ACT model.

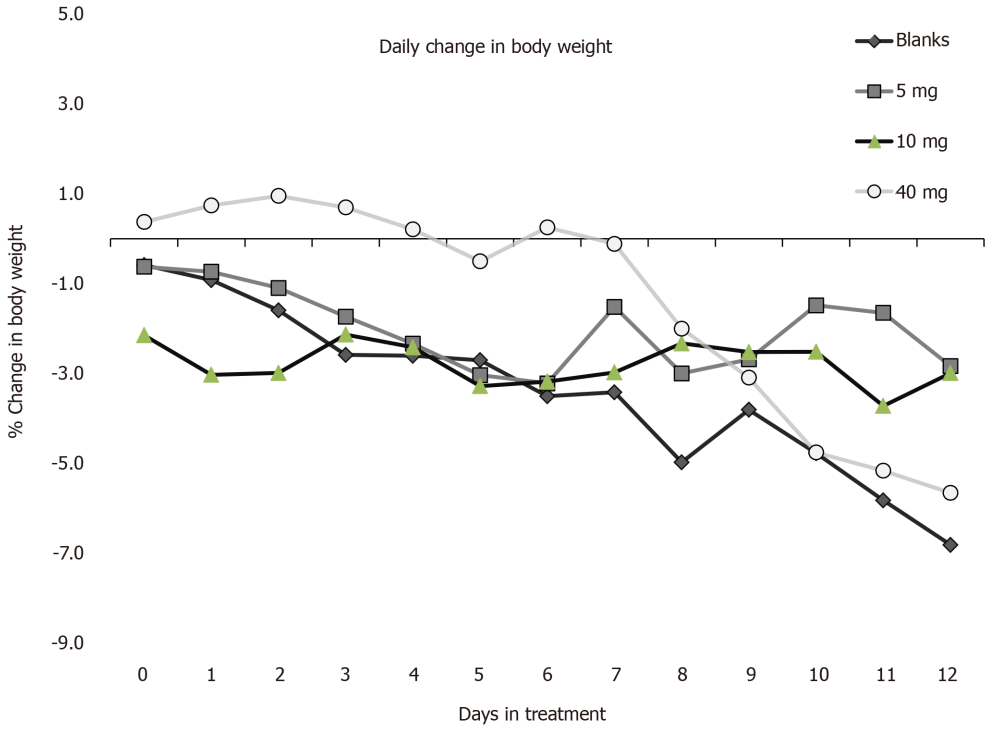


Figure 5 Therapeutic activity of transforming growth factor β loaded particles (TPX6001) in the SCID mouse adoptive CD4+ CD25- T-cell transfer model of inflammatory bowel disease. Mice (*n* = 6-9 per group) with established disease were weighed (day 0) and fed transforming growth factor β1 microspheres (5, 10, or 40 mg/mouse), or blank microspheres (40 mg/mouse) in 0.2 ml water 3 times per week for 2 wk. Mice were monitored for overall disease score and weighed 3 times per week for two weeks. Mice were sacrificed 2 d after the last dose, serum taken, colons weighed and measured; and colons samples prepared for histological analysis (five randomly selected sections from each mouse). Data are expressed as % change in body weight relative to day of first treatment. 5 and 40 mg/mouse TPX6001-treated groups were significantly different (*P* = 0.01 on days 3-12) from animals treated with blank microspheres.

Multiple (28 d) oral treatments (thrice weekly) with either TreXTAM or encapsulated TGFβ were safe and well tolerated at the highest doses tested

For both TreXTAM and PLA encapsulated TGFβ GLP pharmacokinetic studies, full industry standard toxicology analyses, including clinical observations clinical pathology, necropsy, histopathology and ophthalmology, were also performed on both male and female animals. There were no statistically significant differences in body weights or weekly food intake among groups, and no significant organ weight changes. There were no test article-related histopathological or other findings and no fibrosis was observed with even the highest doses at the end of treatment (Day 28) or at the end of a 56-d recovery period (data not shown). Encapsulated TGFβ, when given alone was as safe and as well tolerated as the TreXTAM combination.

DISCUSSION

We report here that oral TreXTAM produced a surprising and dramatic decrease in serum and colonic TGFβ levels. While we could not directly demonstrate simultaneous delivery of both drug substances to gut tissues, our *in vitro* and pharmacodynamics observations suggest that it was achieved. In animals given either TreXTAM or PLA encapsulated TGFβ (TPX6001) alone 3 times per week for 25 d, we observed dramatically lower serum TGFβ compared to the same animals before dosing. We also found evidence for a transient benefit of oral TPX6001, at least in terms of weight loss attenuation, in the murine adoptive cell transfer (ACT) model of IBD.

TreXTAM is being developed as treatment for Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are chronic disorders of the GI tract causing significant morbidity for over 1.4 million Americans[38]. An appreciation of common inflammatory pathways led to the joint designation "IBD". Symptoms include diarrhea, nausea, abdominal pain, weight loss, increased risk for colorectal cancer[39] and can be fatal[40]. Although etiologies are incompletely understood, genetic, immunologic and environmental factors all make significant contributions[38,41]. Human and animal studies implicate abnormal responses to commensal microflora and perturbed local

immune homeostasis[38,39,41]. 'Biologics', macromolecules that target inflammatory lymphocytes or the cytokines they produce[42] have emerged as a new class of highly effective treatments. However, an estimated 30% of patients will not respond and of those who initially respond, 50% relapse within a year. A more recent review indicates only modest impact on surgical intervention rates[43]. The need for novel, targeted therapies remains acute. TreXTAM aims to address that need by taking advantage of the synergistic effects of ATRA and TGFβ on the differentiation and stabilization of regulatory T cells[2].

TGFβ is a pleiotropic cytokine with multiple effects on many cell types. It is a key regulator of T-cell biology, impacting thymocyte development, differentiation and effector function[44]. On the one hand, complete loss of TGFβ signaling leads to lymphoproliferative autoimmunity[45-47], on the other hand, systemic administration in microgram doses protects in several autoimmune disease models[48-51]. Unfortunately, TGFβ is also associated with serious side effects, including pulmonary fibrosis[52-55], scleroderma[56], chronic GVHD[57] and glomerulonephropathies[58]. To circumvent these toxicities, local delivery *via* gene therapy has been proposed, but is inconvenient, transitory, imprecise and immunogenic[48,50,59]. There is no means to control signal transcription or translation, dose schedule, release rates or unwanted immune responses. TreXTAM, aims to circumvent this problems by local delivery and reduces systemic exposure of drug substances with the hope of reducing effective doses and toxicities.

Because of the known fibrotic effects of TGFβ, exacerbation of fibrosis in the context of IBD was a serious concern of oral TreXTAM treatment. The results reported here suggest the opposite might be true, especially in colon, where TreXTAM reduced endogenous TGFβ levels. 28-d TreXTAM repeat dosing studies in rats, like the one reported here for encapsulated TGFβ alone, showed no TreXTAM induced fibrosis in any organ including small intestines and colon (data not shown). Further, we tested TreXTAM, both in healthy mice and in SCID animals with CD4+ CD25- induced colitis, for up to 8 wk, and likewise, found no increases in fibrosis in any organ (Auci *et al*, unpublished observations). Considering the results reported here, oral treatment with TreXTAM, or even treatment with encapsulated TGFβ alone, may be useful to stimulate autocrine negative feedback and reduce TGFβ levels, to prevent IBD associated fibrosis.

Our inability to detect increased TGFβ in small intestine and MLN after TreXTAM treatment may be due to insignificant amounts of TGFβ delivered despite effective particle uptake in the Peyer's patches and MLN(7). This may relate to the failure of the particles to reach the colon or rapid degradation and/or deactivation of the released TGFβ. Uptake by other tissues, binding to cell surface proteins or other factors, as well as the potential conversion of TGFβ1 to TGFβ2, 3 or its latent form, would have prevented an increase from being detected. Perhaps most surprisingly, we observed a highly significant TreXTAM-associated decrease (approximately 50%) of active TGFβ in the colon. While this may relate to effects of the particles themselves, a more intriguing possibility involves ATRA amelioration of TGFβ expression and signaling[60]. Several studies report ATRA decreases TGFβ levels and/or signaling in various tissues[61-64]. ATRA modification of TGFβ signaling may also help explain the lack of treatment associated fibrosis observed in our previous studies[1]. Reduction of endogenous TGFβ in colon and its simultaneous delivery to immune structures such as Peyer's patches and MLN may contribute to the TreXTAM-associated benefits in models of IBD. Like observations in colon, decreases in systemic TGFβ were observed when the encapsulated cytokine was delivered with ATRA in the form of TreXTAM, but also when given alone. Therefore, at least the systemic attenuation of TGFβ levels do not require ATRA and can be achieved with just the encapsulated cytokine. The role of TreXTAM in IBD, including its prophylactic and/or therapeutic usefulness for Crohn's disease and/or colitis, awaits further studies in various models aimed at determining the contribution each component plays in the efficacy observed.

Our finding of higher levels of TGFβ in female *vs* male rats is reminiscent of other studies in humans[65] and in non-human primates, where TGFβ levels were found to be higher in young females as compared to males. Interestingly, TGFβ levels decreased with age in females, and increased with age in males, suggesting effect of sex hormones[66]. The wide literature describing activities of TGFβ in the context of autoimmunity and infection has already been extensively reviewed[67], and its consideration is beyond the scope of this work. Suffice to say that an important intersection for the cross talk between TGFβ signaling pathways and sex hormones may lie at the generation and stabilization of regulatory T cells.

Reminiscent of our observations with TreXTAM in tissue, we found that oral TPX6001 when given alone, without ATRA, also reduced serum levels of the

endogenous cytokine. The mechanism(s) by which oral treatment with encapsulated TGFβ could lead to reduction in systemic and tissue levels remain unknown. They may relate to synthesis or release of mediators by cells, increased uptake and/or deactivation by other tissues[68] and/or effects on pathways specific to immune structures of the gut. TGFβ is synthesized as an inactive precursor, a complex consisting of a TGFβ dimer, the latency-associated protein, and latent TGFβ binding protein[69]. Before TGFβ can exert its biological effects, both must be dissociated. Therefore, our findings may also relate to specific activation/deactivation pathways, which may be controlled by the gut. It is also possible that our findings relate to switching between immunologically (ELISA) distinct isoforms of TGFβ (1, 2 or 3)[70]. The potential biological significance of such switching is unclear.

To our knowledge, we were the first to administer PLA encapsulated TGFβ *via* the oral route[1]. Our preliminary observations in the ACT model of IBD suggest only a transient benefit of oral TPX6001 treatment. However, several studies report activities of oral TGFβ, when given as an intact protein. Shiou *et al*[71] reported that oral administration of TGFβ (30 ng/mL) suppressed pro inflammatory cytokine production (including IL-6 and IL-8) in the gut of rat pups. The suppression was associated with suppressed NF-κB signaling. Systemic TGFβ levels were not measured. An earlier publication by Ando *et al*[72] reported increased serum TGFβ in mice after oral administration of the intact protein. Those studies also reported enhancement of oral tolerance. Additional studies in the ACT model, as well as other models of acute and chronic IBD, will be necessary to fairly evaluate the therapeutic potential of oral TPX6001 when given alone in IBD and perhaps also in other specific clinical situations where increasing TGFβ levels are pathogenic, for example against certain challenging forms of breast cancer[73]. Such studies are subjects of forthcoming work from our laboratories.

CONCLUSION

These observations suggest a negative feedback mechanism in the gut whereby local delivery of TGFβ results in reduced local and systemic levels of the active form of TGFβ. Our findings suggest potential clinical implications for use of encapsulated TGFβ, perhaps in the context of IBD and/or other instances of fibrosis and/or pathological TGFβ signaling.

ARTICLE HIGHLIGHTS

Research background

TreXTAM® is a combination of transforming growth factor beta (TGFβ) and all trans retinoic acid (ATRA) microencapsulated for oral delivery to immune structures of the gut. It is in development as a novel treatment for inflammatory bowel disease (IBD).

Research motivation

When given together, ATRA and TGFβ signals synergize in promoting the differentiation and stabilization of regulatory T cells.

Research objectives

This is a completely novel strategy for the treatment of IBD, as no similar products currently exist. TreXTAM would represent an entirely novel IBD treatment modality.

Research methods

During TreXTAM development, we studied TGFβ pharmacokinetics after oral administration of TreXTAM, or after the encapsulated cytokine (TPX6001) was given alone, without ATRA. This is required for combinatorial products.

Research results

We made the surprising discovery that oral administration of TreXTAM dramatically reduced TGFβ levels in colon and in blood, to below baseline levels. When encapsulated TGFβ (TPX6001) was given alone, three times a week for 25 d, we likewise observed serum TGFβ decreases below baseline (untreated) levels. Oral treatment with TPX6001 alone transiently ameliorated weight loss in the murine

adoptive cell transfer model of IBD, chosen because it recapitulates regulatory T cell immunology associated with disease.

Research conclusions

These observations suggest a negative feedback mechanism in the gut whereby local delivery of TGFβ results in reduced local and systemic levels of the active form of TGFβ. This finding suggests potential clinical implications for use of encapsulated TGFβ in the context of IBD and/or pathological TGFβ signaling.

Research perspectives

Additional studies in the ACT model, as well as other models of acute and chronic IBD, will be necessary to fairly evaluate the therapeutic potential of oral TreXTAM, as well as TPX6001 when given alone in IBD, autoimmune diseases, and perhaps also in other specific clinical situations where increasing TGFβ levels are pathogenic, for example against certain challenging forms of breast cancer. Such studies are subjects of forthcoming work from our laboratories.

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ORIGINAL ARTICLE

Basic Study

Bowel adhesion and therapy with the stable gastric pentadecapeptide BPC 157, L-NAME and L-arginine in rats

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Abstract

BACKGROUND

After parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats, there is failed vasculature, and finally, increased adhesion formation. We hypothesized that unlike nitric oxide (NO)-agents, L-NAME and/or L-arginine, the application of the stable gastric pentadecapeptide BPC 157 with its most recent vascular effects ("vascular recruitment") means attenuated bowel adhesion formation and NO- and malondialdehyde (MDA)-tissue values.

AIM

To focused on the bowel adhesion and the therapy with the BPC 157, its most and application of NO-agents.

METHODS

Along with defect creation, medication was (1) during surgery, once, at 1 min after defect creation as an abdominal bath (1 mL/rat), BPC 157 (10 μg/kg, 10 ng/kg, 1 mL/rat), an equivolume of saline, L-NAME (5 mg/kg), L-arginine (200 mg/kg) alone and/or combined. Alternatively, medication was (2) intraperitoneally once daily, first application at 30 min after surgery, last application 24 h before assessment at d 7 or d 14. As a postponed therapy to pre-existing adhesion (3), BPC 157 (10 μg/kg, 10 ng/kg intraperitoneally, 1 mL/rat) was given once daily since d 7.

RESULTS

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The recovery effect of the BPC 157 regimens goes with the presence of abundant vascular vessels in and near the defect, which occurs rapidly. Lastly, also applied as post-treatment, BPC 157 creates attenuated adhesions, minimal or no adhesion. Contrarily, NO-agents have diverse initial and final effects: The initial weakening of blood vessel disappearance and finally, severe worsening of adhesions (L-NAME) *vs* the initial weakening of blood vessel disappearance and finally, attenuation of adhesions formation (L-arginine), which counteract each other response given together. Importantly, BPC 157 maintains its beneficial effect also when given with NO-agents (L-NAME + BC 157; L-arginine + BPC 157; L-NAME + L-arginine + BPC 157). Finally, with respect to the increased NO- and MDA-values-adhesion tissue formation relation, unlike diverse effect of the NO-agents, the BPC 157 application effect regularly combines decrease on the increased NO- and MDA- values and the beneficial outcome (less adhesion formation).

CONCLUSION

BPC 157 therapy can be suited for the realization of the peritoneal defect healing with minimal or no adhesion formation.

Key Words: Abdominal wall defect; Adhesions; BPC 157; Vascular recruitment; Nitric oxide-agents; Rats

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Core Tip: After parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats, there is failed vasculature, and finally, increased adhesion formation. Unlike nitric oxide-agents, L-NAME and/or L-arginine diverse effects, the application of the stable gastric pentadecapeptide BPC 157 with its most recent described vascular effects ("vascular recruitment") recovers abundant vascular vessel presentation in and close to the defect, which occurs rapidly. Finally, BPC 157 attenuated bowel adhesion formation and nitric oxide- and MDA-tissue values. Thus, BPC 157 therapy can be suited for the realization of the peritoneal defect healing with minimal or no adhesion formation.

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INTRODUCTION

We employed an excision of the parietal peritoneum with the underlying superficial layer of muscle tissue, and assessed the consequences thereof. There was a downhill course, from the very early events to massive bowel adhesion. However, recovery was achieved with the stable gastric pentadecapeptide BPC 157 and nitric oxide (NO) system involvement[1-12]. This may be due to the rapid recovery of failed blood vessels after induction of the initial injury. Such therapy has had beneficial effects in previous studies on vascular occlusion[13-19]. This recovery effect may be due to its effect on occluded vessels; it activated bypassing pathways, thereby counteracting occlusion consequences[13-19]. This was described[13-17] in the treatment of deep vein thrombosis and colitis ischemia/reperfusion, duodenal venous congestion and cecum perforation[13-16], and bile duct ligation-induced liver cirrhosis and portal hypertension rat injuries[17]. Recently, this recovery was also shown in the rescue of quite complex severe circumstances, *i.e.*, Pringle manoeuvre ischemia and reperfusion (portal triad temporary occlusion) as well as Budd-Chiari syndrome (induced by the suprahepatic occlusion of the inferior vena cava)[18,19]. The cytoprotective impact of BPC 157 on the endothelium[1-12] may have special importance. Namely, the peritoneal lining of the cavity serves as a conduit for blood vessels that are markedly disturbed by parietal

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peritoneum injury[20]. This study assessed the normalisation of the level of malondialdehyde (MDA) and NO in tissue and the effect of the NO agents L-NAME and/or L-arginine, given alone and/or together, as confirmation[13-19].

There, BPC 157 (GEPPPGKPADDAGLV, M.W. 1419, LD1 not achieved, implemented in inflammatory bowel disease trials and now multiple sclerosis[1-12]) as a prototype of the more potent cytoprotective agent (*i.e.*, it also counteracts pre-existing lesions[1-12]), may be an agent to counteract adhesion formation, as a therapy. As an anti-ulcer peptide, stable in human gastric juice, it is designed to be a novel mediator of Robert's cytoprotection[1-12] (epithelium/endothelium protection [1-12]). Likely, due to this effect (epithelium/endothelium protection[1-12] as a shared class effect of cytoprotective agents), it is effective in the entire gastrointestinal tract[1-12], and counteracts the bowel adhesion formation, which is induced by different procedures[21-33]. Thereby, with an apparent vessel-adhesions related effect, BPC 157 corroborates all these vascular effects, and enhances vascular rescue after injury in particular[13-19], by counteracting adhesion formation. Also, it interacts with the NO system in various species and models[1-12], and counteracts the adverse effect of both L-NAME and L-arginine, given as individual agents or combined[1-12].

The NO system is implicated in cytoprotection[1-12] and adhesion formation[34]. However, there has been no study of the effect of L-NAME (ulcerogenic) and/or L-arginine (beneficial)[1-12] application on the immediate presentation of the blood vessels as a hallmark (vessel empty/disappeared; vessel filled/reappeared), during and after parietal peritoneum injury.

Therefore, it may be that formation of adhesion as an effect on the NO system suggests the beneficial effect related to the NO system. Thus, BPC 157, L-NAME and L-arginine were administered alone or in combination. The medication BPC 157 was applied either once, at 1 min after defect creation as an abdominal bath (1 mL/rat) to assess the initial post-injury course, blood vessel presentation, or to assess the subsequent course leading to adhesion formation intraperitoneally once daily, with the first application at 30 min after surgery and the last application 24 h before the assessment. Alternatively, from day 7, BPC 157 was given intraperitoneally once daily as a postponed therapy for a pre-existing adhesion. At these points in the injured tissue, levels of MDA and NO were assessed, and the results showed the NO levels and oxidative stress (MDA) were normalised in injured tissue.

MATERIALS AND METHODS

Animals

Male Albino Wistar rats, 200 g, were randomly assigned (7 rats per group) and used for the experiments, which were approved by the local ethics committee. The perforation procedure was performed in rats that had food and water ad libitum before the procedure and until the end of the experiment. The animal protocol was designed to minimise pain or discomfort to the animals. The animals were acclimatised to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 wk prior to experimentation. All animals were euthanised by a barbiturate overdose (intraperitoneal injection, 500 mg/kg thiopental sodium) prior to tissue collection.

Drugs

The pentadecapeptide Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val, M.W. 1419, called stable gastric pentadecapeptide BPC 157, part of the sequence of the human gastric juice protein BPC, freely soluble in water and 0.9% NaCl at pH 7.0 (Diagen, Ljubljana, Slovenia), prepared as described previously[1-12]. The peptide had 99% purity by high-pressure liquid chromatography, with the 1-des-Gly peptide as a biologically inactive impurity[1-12]. L-NAME and L-arginine were commercially purchased (Sigma, United States).

Procedure and adhesion assessment

Under deep anaesthesia (thiopental (Rotexmedica, Germany) 40 mg/kg, diazepam (Apaurin, Krka, Slovenia; 10 mg/kg), excision of the parietal peritoneum (1 cm × 2 cm, 2 cm right of the median, 3 cm laparotomy) with an underlying superficial layer of muscle tissue was performed in rats. BPC 157 (10 μg/kg, 10 ng/kg, 1 mL/rat), or an equivalent volume of 0.9% NaCl were applied, with L-NAME (5 mg/kg) or L-arginine (200 mg/kg) given alone or in combination with BPC 157 at both concentrations. The medication was applied either once at 1 min after defect creation as an abdominal bath

(1 mL/rat), or intraperitoneally once daily, with the first application at 30 min after surgery and the last application 24 h before the assessment. Alternatively, starting from d 7, BPC 157 (10 μg/kg, 10 ng/kg intraperitoneally, 1 mL/rat) was given once daily as a postponed therapy for a pre-existing adhesion. Immediately after defect creation, using a USB microscope camera (Veho discovery VMS-004D-400x USB microscope; Veho®, United Kingdom), we recorded the presentation of blood vessels, *i.e.* augmentation/reduction[13-19] (emptied/disappeared; refilled/reappeared) and assessed at the end of the next 10-min period as total% of vascular vessels presentation in and close to the defect concerning the point immediately before therapy. Also, using a USB microscope camera (Veho discovery VMS-004D-400x USB microscope; Veho® ,United Kingdom) at d 7 or d 14, adhesions were scored using Mazuji's classification [no adhesion (score 0); very small, irregular adhesion (score 1); easily separable medium intensity adhesion (score 2); intense, not easily separable regular adhesion (score 3); very intense, not easily separable, homogenous adhesion][35].

Immediately after culling the animals, tissue specimens were fixed in buffered formalin (pH 7.4), for 24 h, dehydrated and embedded in paraffin wax. The samples were stained with haematoxylin–eosin. Tissue injury was evaluated microscopically by a blinded examiner.

Oxidative stress in adhesion tissue

At 7 d or 14 d post-injury, oxidative stress in the tissue samples (1 cm^2 around the defect) was assessed by quantifying thiobarbituric acid reactivity as MDA equivalents. Trichloroacetic acid was added to homogenise the tissue samples, which were then centrifuged (3000 rpm, 5 min), and the supernatant was collected. Thereafter, 1% thiobarbituric acid was added, and the samples were boiled (95 °C, 60 min). The tubes were kept on ice for 10 min, and the absorbance was determined at the wavelengths of 532 and 570 nm. The concentration of MDA was read from a standard calibration curve plotted using 1,1,3,3′-tetra-ethoxy propane. The extent of lipid peroxidation was expressed as MDA using a molar extinction coefficient for MDA of 1.56×10^5 mol/L/cm. The results are expressed in nmol/mg of protein[13-19].

Nitric oxide determination in adhesion tissue

At 7 d or 14 d post-injury, we determined the NO levels in cecum tissue samples using the Griess reaction (Griess Reagent System, Promega, United States). Sulphanilamide was then incubated with the homogenised tissue, and then, N-1-naphthyl ethylenediamine dihydrochloride was added. The Griess reaction is based on a diazotisation reaction in which acidified nitrite reacts with diazonium ions and, in a further step, are coupled to N-1-naphthyl ethylenediamine dihydrochloride, forming a chromophoric azo derivate. The absorbance was measured at 540 nm using a sodium nitrite solution as the standard. NO levels are reported in μmol/mg protein. The protein concentrations were determined using a commercial kit (BioRad Protein DR Assay Reagent Kit, United States)[13-19].

Statistical analyses

Statistical analysis was performed by parametric one-way ANOVA with the post-hoc Newman-Keuls test and non-parametric Kruskal-Wallis and subsequent Mann-Whitney *U*-tests to compare between groups. The values are represented as mean ± SD and minimum/median/maximum. Results with $P < 0.05$ were considered significant. The statistical methods of this study were reviewed by Bozo Radic, MD, PhD at Clinical Hospital Dubrava.

RESULTS

In this study, the focus was on the possible therapeutic effect of the application of the stable gastric pentadecapeptide BPC 157, and NO agents, *i.e.* L-NAME and L-arginine, after the creation of a defect by the excision of the parietal peritoneum with an underlying superficial layer of muscle tissue in rats. Therefore, this study reveals the formation of adhesions which could extensively occupy and obstruct the abdominal wall and intestine and pelvic organs (Figures 1-5 with respect to the initial defect creation, and the early presentation of blood vessels in and close to the peritoneal defect (Figures 6 and 7). Support was obtained from the presentation of MDA- and NO tissue levels in adhesion tissues (Figure 8).

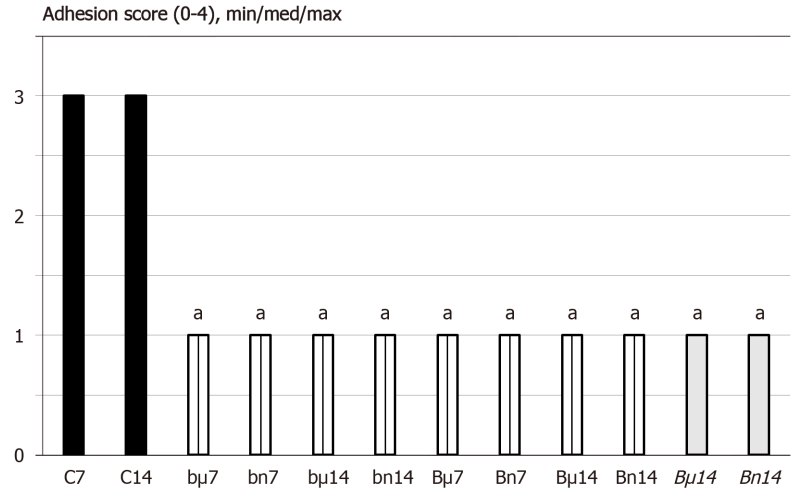


Figure 1 At 7 d (7) or 14 d (14) post-injury, we assessed the adhesion score (0-4), min/med/max. Medication (/kg), was BPC 157 [10 μg (μ) or 10 ng (n)] (b - 1 mL bath/rat at abdominal cavity, B – once daily intraperitoneally, first application at 30 min following surgery, last 24 h before sacrifice; B once daily intraperitoneally, first application at day 7 following surgery, last 24 h before sacrifice) or saline(controls)(C) (bath equal volume, or once daily intraperitoneally (since not different results are shown together). $^{a}P < 0.05$ at least *vs* control.

Adhesions

Controls: The induction of particular defect resulted in the formation of a large adhesion, which occupied and obstructed the abdominal wall, intestine and pelvic organs, as observed on d 7 and d 14 in all controls (Figures 1-5).

BPC 157: On d 7 and 14, after treatment with BPC 157, the degree of adhesion was minimal or absent. To emphasise the conclusive point (less or no adhesion formation), medication (/kg), BPC 157 (10 μg or 10 ng) was applied once as a 1 mL bath/rat in the abdominal cavity immediately after the creation of the defect, or once daily intraperitoneally, with the first application at 30 min after surgery and the last 24 h before culling. The final application was given once daily intraperitoneally, with the first application on d 7 following surgery and the last 24 h before culling (Figure 1, Figure 4, and Figure 5).

NO agents: The application of L-NAME resulted in severe worsening. On the contrary, the L-arginine caused the attenuation of adhesion formation. When used together, L-NAME and L-arginine antagonised each other, leading to adhesion formation similar to control animals (Figure 2).

BPC 157 and NO agents: Together, all these treatments (L-NAME + BPC 157, L-arginine + BPC 157, L-arginine + L-NAME + BPC 157) produced a benefit similarly to the application of BPC 157 alone (Figure 2).

Microscopic images (Figure 5) are provided along with the gross presentation. After the first week, controls presented oedematous granulation tissue, covering large areas of the serosa, whereas in BPC 157-treated rats, far smaller areas were covered with denser and more mature granulation tissue with vessels appearing to be more mature and better formed. After two weeks, large areas of still poorly organised granulation tissue invading the bowel and abdominal wall were obvious in control animals. In BPC 157-treated rats, a young connective tissue scar had formed in small areas, with poor invasion into the bowel serosa/subserosa leading to very limited and non-strangulating adhesions.

Vascular presentation

Controls: Along with defect creation, since the blood vessels quickly became empty, they rapidly disappeared close to the defect.

BPC 157: Recruitment of blood vessels appeared after or with BPC 157 treatment as an abundant vascular presentation within and close to the defect, which occurred rapidly.

NO agents: The application of L-NAME and L-arginine led to the slowing of blood vessel disappearance. When used together, L-NAME and L-arginine antagonised each other, showing similar vessel presentation as with the application of the saline in control animals.

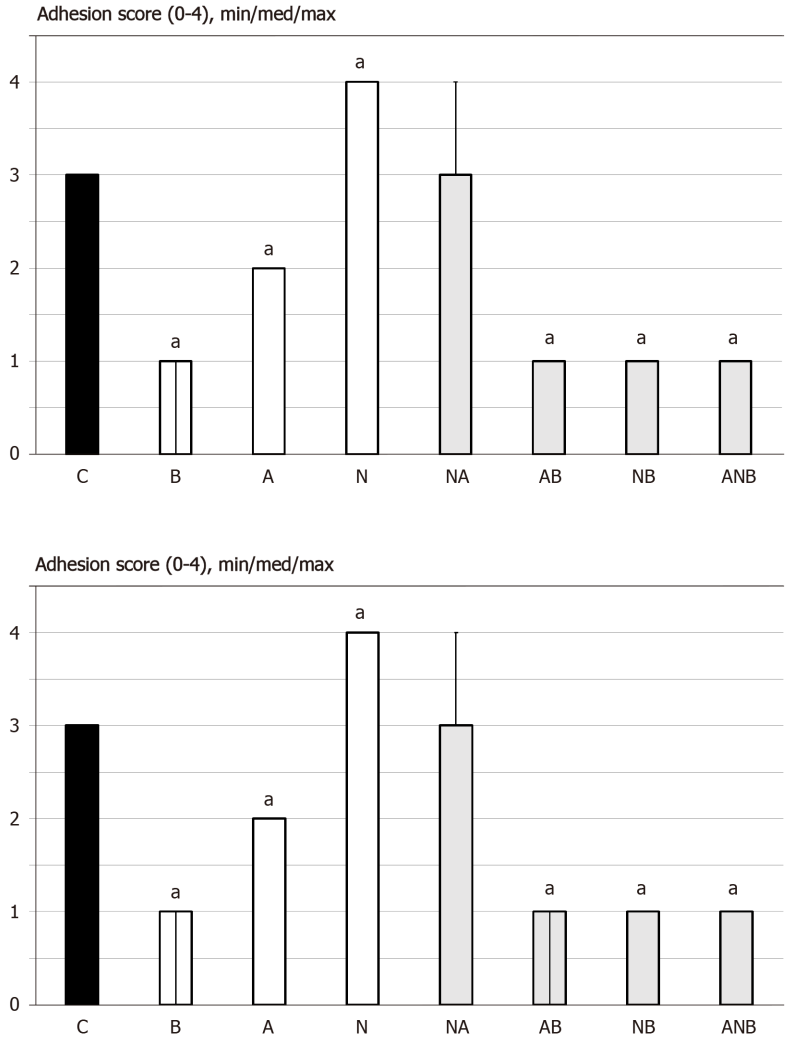


Figure 2 At 7 d or 14 d post-injury, we assessed the adhesion score (0-4), min/med/max. Medication (/kg, 1 mL bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations (L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls) (C). $^{a}P < 0.05$ at least *vs* control; BPC 10 ng presented adhesion score 1/1/1 with L-arginine (L-arginine + BPC 157); Score 1/1/1 with L-NAME (L-NAME + BPC 157); Score 1/1/1 with L-arginine and L-NAME (L-arginine + L-NAME + BPC 157), $^{a}P < 0.05$ at least *vs* control.

BPC 157 and NO agents: Given together, these treatments (L-NAME + BPC 157, L-arginine + BPC 157, L-arginine + L-NAME + BPC 157) produced a benefit in much the same way as the application of BPC 157 alone.

NO/oxidative stress

Controls: Along with adhesion formation, increased values appeared at day 7, while on day 14 the values, particularly NO were not as high.

BPC 157: With BPC 157 treatment, the values of NO and MDA appeared to be normalised (decreased) at both time points.

NO agents: The application of L-NAME reduced NO values, and L-arginine increased NO values at day 7 and d 14. When given together, L-NAME and L-arginine antagonised each other. Interestingly, compared with the controls at day 7 and day 14, both L-NAME and L-arginine increased MDA values, and when given together, L-NAME and L-arginine antagonised each other.

BPC 157 and NO agents: Given together, all of these treatments (L-NAME + BPC 157, L-arginine + BPC 157, L-arginine + L-NAME + BPC 157) produced a counteracting effect on both increased NO and MD levels, much like the application of BPC 157 alone.

Thus, in rats with the parietal peritoneum and adjacent muscle removed, we

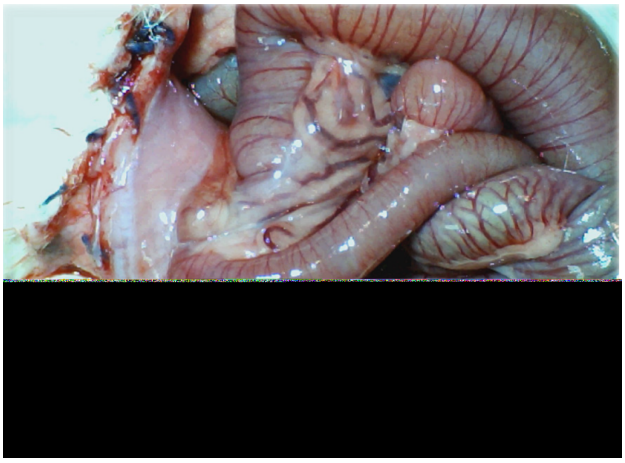


Figure 3 Adhesion formation (Control rats). Illustrative presentation of adhesion formation leading to subileus presentation at day 14 post injury.

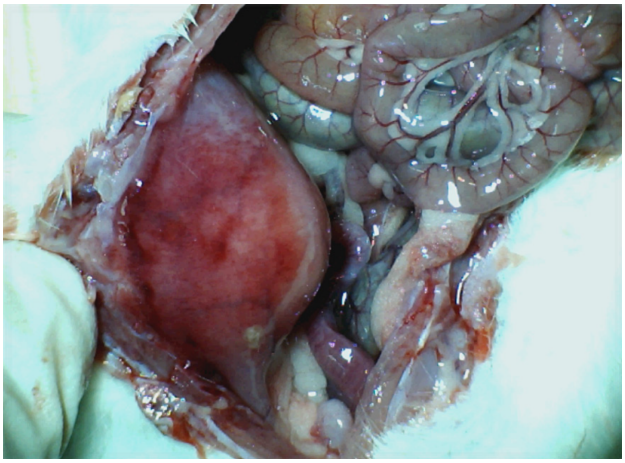


Figure 4 Adhesion formation. BPC 157 rats. Illustrative presentation of the abdominal wall healing without adhesion formation at d 14 post injury.

demonstrated the particular combination of early to final response, which was affected by the application of the stable gastric pentadecapeptide BPC 157, L-arginine and L-NAME, administered alone or together. Of note, only with the BPC 157 application was a beneficial outcome observed, *i.e.* less adhesion formation and suppressed NO and MDA values.

DISCUSSION

In this study, we performed an excision of the parietal peritoneum with the underlying superficial layer of muscle tissue, and assessed the downhill consequences thereof with the rapid recovery of the failed blood vessels after induction of the initial injury. Recovery with the stable gastric pentadecapeptide BPC 157 involved the NO system[1-12], as shown with the related effects of L-arginine and/or L-NAME.

This study on abdominal wall injury has basic relationships, such as vascular failure (vessels, one empty, "disappear") at a very early post-injury time point, followed by a failure outcome (*i.e.*, the formation of extensive adhesions). MDA values and NO levels increased after the initial abdominal wall defect was made[36,37], and increase during subsequent adhesion formation[36,37]. On the contrary, the possible recovery effect is that the filled vessels "reappeared" and adhesion formation was attenuated. Suppression of NO and MDA levels matched with the beneficial outcome (less adhesion formation. Only BPC 157 therapy in the damaged abdominal wall led to this combined beneficial effect. The very rapid activation of the blood vessels in the initial defect likely circumvented the subsequent negative chain of events and resulted in the resolution of adhesion and obstruction. Similar effectiveness was observed after

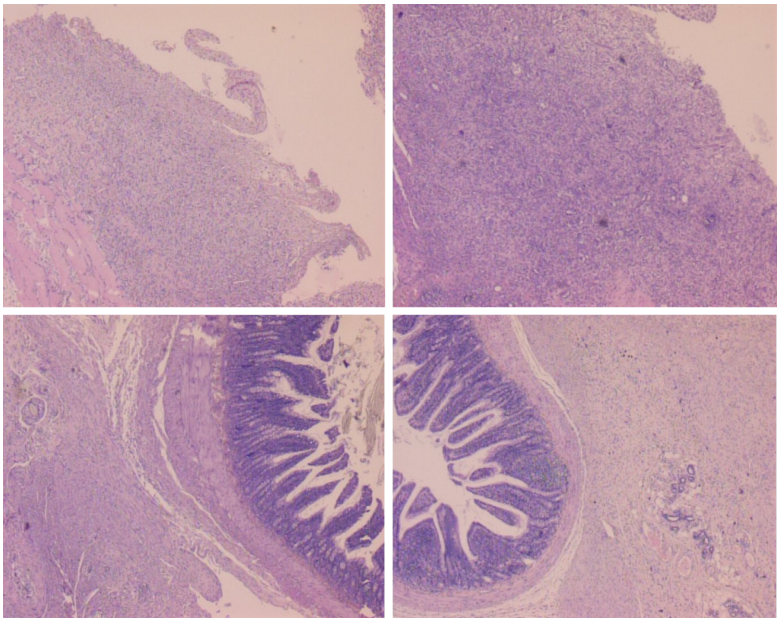


Figure 5 Histological assessment after 7 d (upper) and 14 d (low), in rats subjected to the excision of the parietal peritoneum with underlying superficial layer of muscle tissue, controls [upper left (7 d), low left (14)] and BPC 157-treated rats [upper right (7 d), low right (14 d)]. 7 d. Upper, left, controls. Edematous, relatively poorly formed granulation tissue, covering large areas of serosa. Upper, right, BPC 157. By far smaller areas covered with more dense and mature granulation tissue with vessels appearing more mature and better formed. 14 d. Low, left, controls. After two weeks large areas of still poorly organized granulation tissue invading the bowel and abdominal wall. Low, right, BPC 157. Young connective tissue scar is formed in smaller areas, with poor invasion into the bowel serosa/subserosa leading to very limited and non-strangulating adhesions. HE stain, objective × 10.

postponing therapy, as this counteracting effect occurred even with pre-existing adhesions (adhesion reduction from day 7 to d 14).

The effect of BPC 157 is quite persistent, since BPC 157 + L-NAME, BPC 157 + L-arginine and BPC 157 + L-NAME + L-arginine rats showed similar outcomes as BPC 157-rats. Thus, this counteraction occurred when challenged with nitric oxide synthases (NOS) blockade (L-NAME; less vessel presentation within and close to the defect, more adhesions and higher NO and MDA values) as well as NOS overstimulation (L-arginine; less vessel presentation within and close to the defect, fewer adhesions but higher NO and MDA values). Thus, the NO system was immobilised when L-NAME and L-arginine counteracted each other's effect. In these rats, which exhibited a disease course similar to the controls, in any circumstance, early or late, additional BPC 157 co-application (L-NAME + L-arginine + BPC 157) re-established the counteraction, as did BPC 157 application (BPC 157 + L-NAME-, BPC 157 + L-arginine-; BPC 157 + L-NAME + L-arginine-rats ≈ BPC 157-rats). Important for the establishing BPC 157-NO relationships (for review see, see[1-12]), L-NAME (NOS blockade) *vs* L-arginine (NOS over-stimulation) *vs* the combination (NO system immobilisation) were able to fully highlight the particularities of the NO system[1-12]. Thus, with supportive evidence, it is conceivable that BPC 157 therapy mitigates the disturbances caused by parietal peritoneum and adjacent muscle removal and NO agent-induced effects (thereby, particular relations BPC 157 with the healing and NO system). It appears much like vessel recruitment to bypass occlusion and re-establish blood flow in previous occlusion- and ischemia/reperfusion studies[13-19]. BPC 157 therapy may be useful as a combined therapeutic (cytoprotective), while NO agents, *i.e.*, L-NAME and/or L-arginine, are used as an additional control.

Finally, the suggested vessels/adhesions/BPC 157/NO system relationship supports the evidence that the stable gastric pentadecapeptide BPC 157 counteracts bowel adhesion formation related to different procedures[21-28]. Likewise, this may resolve adhesions as an aberrant peritoneal wound healing process[38]. Namely, BPC 157 accelerates the healing of various wounds (*i.e.*, skin[39-42], muscle[43-46], tendon[47-49], ligament[50], bone[51]; ulcers in the entire gastrointestinal tract[14-16,27,28,52-54], corneal ulcers[55], gastrointestinal fistulas[21-25,56]). Consistently, it appears to synchronise healing in various tissues. Also, once the peritoneum is damaged, the coagulation cascade is set in motion[57]; BPC 157 counteracts the whole Virchow triad[13], venous[13,18,19] and arterial thrombosis[18,19,58] and attenuates prolonged bleeding and thrombocytopenia after amputation and anticoagulant use[13,59,60]. Therefore, BPC 157 is likely to interfere when two damaged peritoneal surfaces come into contact with each other. Therefore,

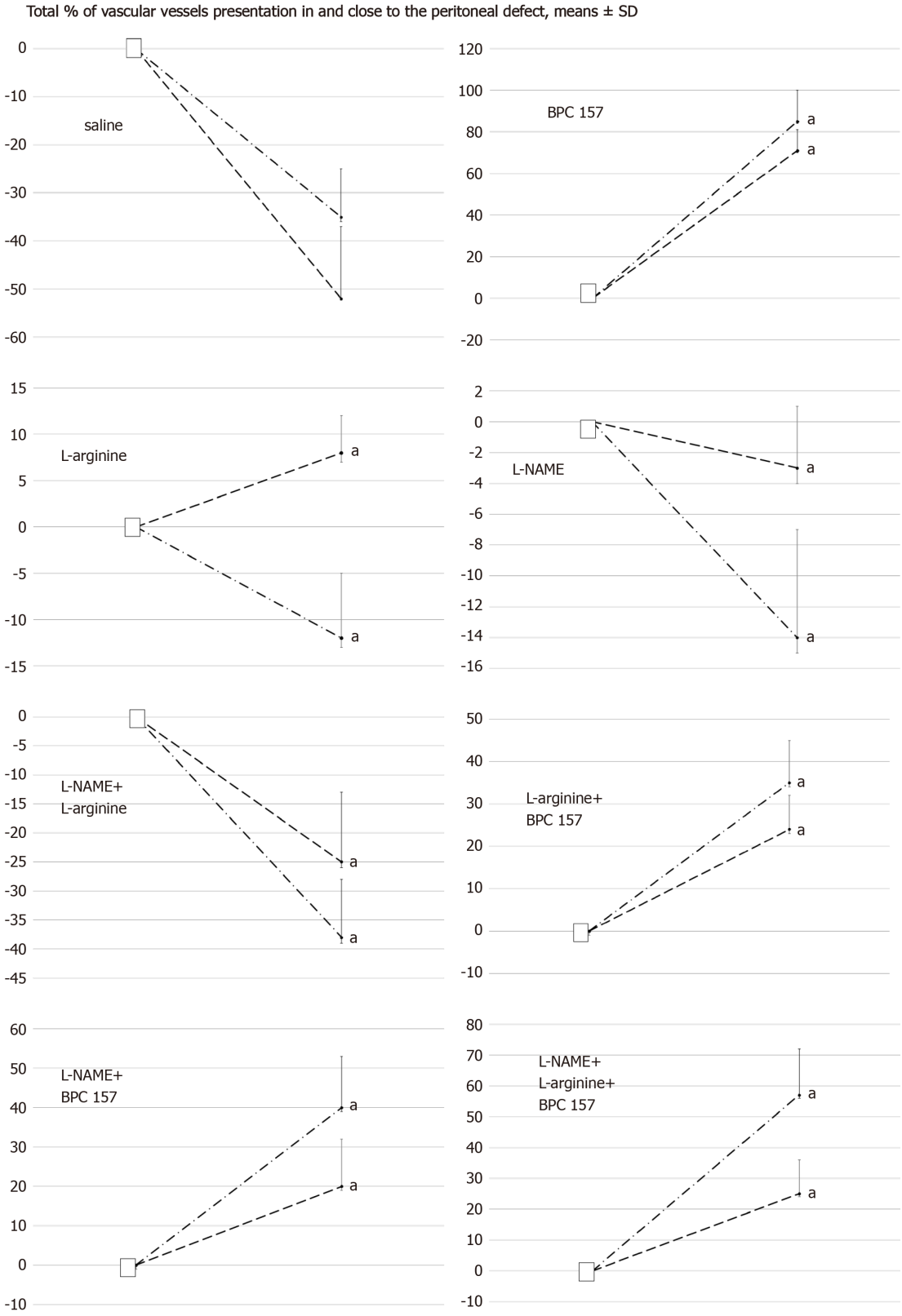


Figure 6 Total % of vascular presentation in (dash) and close to (dash dot) the defect. White squere indicates the values immediately before therapy and full oval the values at the end of the next 10 min. Medication (/kg, 1 mL bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations (L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls) (C). $^{a}P < 0.05$ at least *vs* control; BPC 10 ng presented values 20 ± 8 (in) and 35 ± 8 (close to) with L-arginine (L-arginine + BPC 157); 25 ± 9 (in) and 45 ± 8 (close to) with L-NAME (L-NAME + BPC 157); 20 ± 7 (in) and 55 ± 15 with L-arginine and L-NAME (L-arginine + L-NAME + BPC 157), $^{a}P < 0.05$ at least *vs* control.

by counteracting adhesion formation and reversing existing adhesions, BPC 157 is likely to interfere with and reverse the healing that would result in fusion to form a connection, *e.g.*, an adhesion. Likely, this beneficial effect could be related to 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[36,37].

Interestingly, when given with aspirin, clopidogrel or cilostazol, BPC 157 largely rescues thrombocyte function in rats and does not affect coagulation factors[61]. Additionally, these BPC 157 assets, in particular abundant vascular presentation within and close to the defect as an extension of the original cytoprotective endothelium protection effect[1-12], should be viewed with the stronger angiogenesis effect than the standard anti-ulcer agents[62], along with increased expression of vascular endothelial growth factor (VEGF), and subsequent pathways[3,13,63-67].

In particular, BPC 157 increases the expression and internalisation of VEGFR2, the activation of the VEGFR2-Akt-eNOS signalling pathway without the need for other known ligands or shear stress[63], and heals corneal ulcer and lesions while maintaining corneal transparency[55,68], much like it heals lesions in the entire gastrointestinal tract[14-16,27,28,52-54] and other tissues, gastrointestinal fistulas in particular[21-25,56]. BPC 157 also counteracts tumour cachexia and muscle wasting, characterised by increased pro-inflammatory/pro-cachectic cytokines such as interleukin-6 and tumour necrosis factor-α, and significantly corrects deranged muscle proliferation and myogenesis through changes in the expression of FoxO3a, p-AKT, p-mTOR and P-GSK-3β (mitigating cancer cachexia)[3]. Also, BPC 157 actually counteracts the tumour-promoting effect of VEGF[69].

Finally, as an additional clue, a very recent investigation showed that BPC 157 is a stabiliser of cellular junctions, leading to significantly mitigated indomethacin-induced leaky gut syndrome, *via* increased expression of the tight junction protein ZO-1 (Zonula occludens-1) as well as improved transepithelial resistance[70]. Likewise, the mRNAs of inflammatory mediators [iNOS, interleukin-6, IFN-γ (Interferon gamma) and tumour necrosis factor-α] were inhibited, along with increased expression of HSP (Heat shock proteins) 70 and HSP 90, and antioxidant proteins such as HO-1, NQO-1, glutathione reductase, glutathione peroxidase 2 and GST-pi[70].

CONCLUSION

In summary, adhesion-related obstruction accounts for a considerable number of emergency surgical presentations, and continued research for preventing adhesions is more than desirable[71,72]. Therefore, BPC 157 therapy, establishing the vessels/adhesions/BPC 157/NO system relationship, may be suitable for peritoneal defect healing with minimal or no adhesion formation. Finally, BPC 157[1-12], with lethal dose not achieved, has been implemented in inflammatory bowel disease trials, with a particular effect on vessel presentation and the ability to counteract the harmful effects of NO agents, *i.e.* free radical formation and elevated NO[1-12]. BPC 157 should be further assessed as a therapy to avoid adhesion formation.

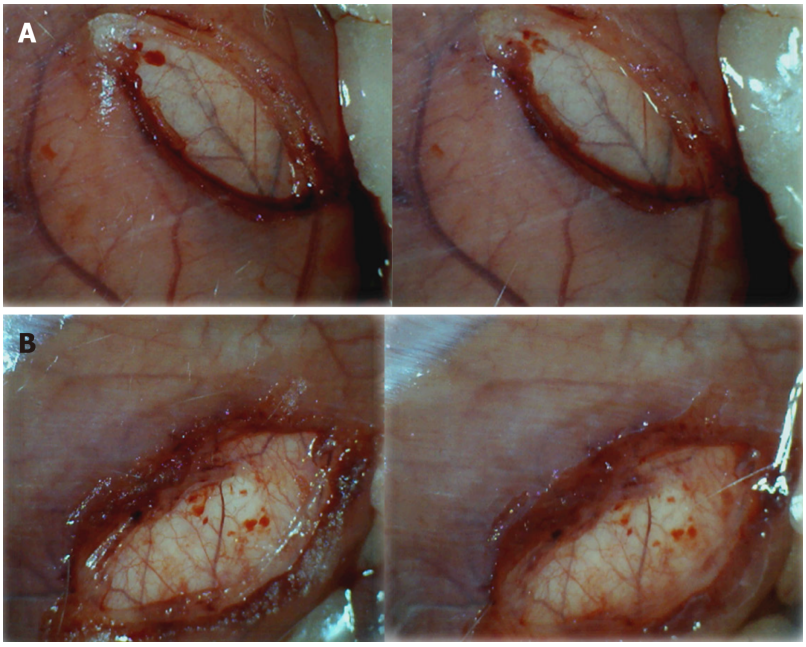


Figure 7 Early vascular presentation in and close to defect, before and immediately after therapy application. A: Control rats. Illustrative presentation of the vessels in and close to the defect immediately before therapy (left), and immediately after therapy application at the abdominal cavity, under saline solution immersion (right). No particular vessel recruitment; B: BPC 157 rat. Illustrative presentation of the vessels in and close to the defect immediately before therapy (left), and immediately after therapy application at the abdominal cavity, under BPC 157 solution immersion (right). Particular vessel recruitment in and close to the defect.

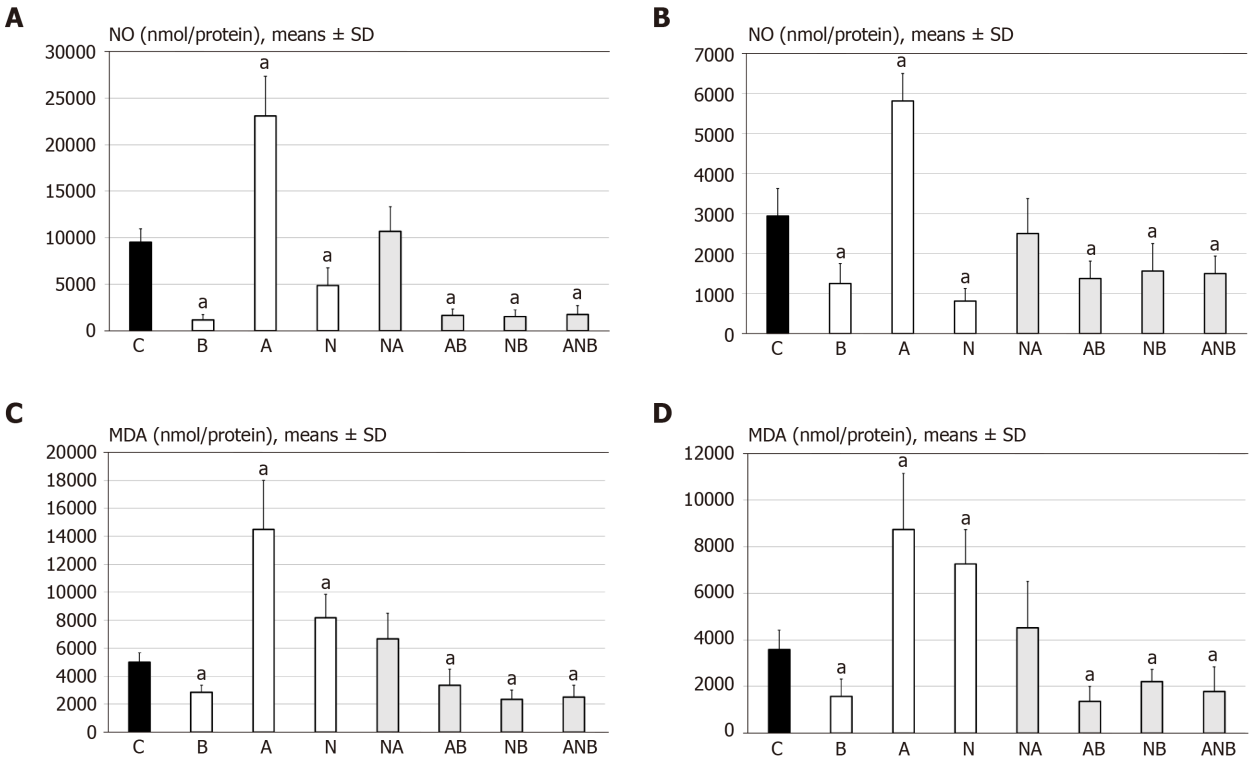


Figure 8 Support was obtained from the presentation of MDA- and NO tissue levels in adhesion tissues. A: At 7 d post-injury, we determined nitric oxide (NO) in adhesion tissue samples using the Griess reaction. Medication (/kg, 10 mL/2 min bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls) (C). $^{a}P < 0.05$ at least *vs* control; B: At 14 d post-injury, we determined NO in adhesion tissue samples using the Griess reaction. Medication (/kg, 10 mL/2 min bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls) (C). $^{a}P < 0.05$ at least *vs* control; C: At 7 d post-injury, we determined oxidative stress in adhesion tissue samples was assessed by quantifying thiobarbituric acid reactivity as malondialdehyde equivalents. Medication (/kg, 1 mL bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls) (C). $^{a}P < 0.05$ at least *vs* control; and D: At 14 d post-injury, we determined oxidative stress in adhesion tissue samples was assessed by quantifying thiobarbituric acid reactivity as malondialdehyde equivalents. Medication (/kg, 1 bath/rat) at abdominal cavity was BPC 157 (10 μg) (B), L-arginine (A) (100 mg), L-NAME (5 mg) alone or in combinations L-NAME + L-arginine (NA), L-arginine + BPC 157 (AB), L-NAME + BPC 157 (NB), L-arginine + L-NAME + BPC 157 (ANB) or saline bath equal volume (controls)(C). $^{a}P < 0.05$ at least *vs* control.

ARTICLE HIGHLIGHTS

Research background

The excise parietal peritoneum with the underlying superficial layer of muscle tissue, since the very early events to the huge bowel adhesion, may represent a model relevant to adhesion-related obstruction and a considerable number of emergency surgical presentation. The downhill course can be counteracted with the recovery by the stable gastric pentadecapeptide BPC 157 and NO-system involvement. This may be the rapid recovery of the failed blood vessels after the initial injury induction. The previous studies of vascular occlusion reveal its particular effect on the occluded vessels, and bypassing of the occlusion, in the therapy of the deep vein thrombosis and colitis ischemia/reperfusion, duodenal venous congestion and cecum perforation, bile duct ligation-induced liver cirrhosis and portal hypertension rat injuries, Pringle maneuver ischemia and reperfusion (portal triad temporary occlusion) as well as Budd-Chiari syndrome (induced by the suprahepatic occlusion of the inferior caval vein). Also, the BPC 157 cytoprotective endothelium impact can have special importance. Namely, the peritoneal lining of the cavity serves as a conduit for the blood vessels that should be markedly disturbed by parietal peritoneum injury. Thus, we considered that BPC 157 therapy is suited for the realization of the peritoneal defect healing with minimal or no adhesion formation.

Research motivation

Adhesion-related obstruction accounts for a considerable number of emergency

surgical presentation, and thereby, whatever mechanisms, the continued research for preventing is more than desirable. Therefore, BPC 157 therapy, establishing vessels-adhesions-BPC 157-NO-system relation, can be suited for the realization of the peritoneal defect healing with minimal or no adhesion formation.

Research objectives

The focus was on the possible therapeutic effect of the application of the stable gastric pentadecapeptide BPC 157, and NO-agents, L-NAME, and L-arginine after the creation of a particular defect by excision of parietal peritoneum with an underlying superficial layer of muscle tissue in rats. The revealing focus was on the formation of the adhesion, which could extensively occupy and obstruct the abdominal wall and intestine and pelvic organs, with respect to the initial defect creation, and the early blood vessels presentation in and close to the peritoneal defect. The support was the presentation of the MDA- and NO-tissue level in adhesion tissues.

Research methods

In the rats with the excised parietal peritoneum with the underlying superficial layer of muscle tissue, we suggest that this formation of adhesion would be an effect on the NO-system and that the BPC 157 beneficial effect would be an effect related to the NO-system.

The medication was BPC 157, L-NAME, L-arginine administered alone or in combinations. The medication was applied either once, at 1 min after defect creation as an abdominal bath (1 mL /rat) to perceive the initial post-injury course, blood vessel presentation (assessed by USB microscope camera, the blood vessels presentation augmentation/reduction to perceive subsequent course leading to the adhesion formation). Application was intraperitoneally once daily, first application at 30 min after surgery, last application 24 h before assessment. Alternatively, from day 7, BPC 157 was given intraperitoneally once daily as a postponed therapy to pre-existing adhesion. At these points in the injured tissue (gross and microscopy assessment), levels of MDA and NO were also assessed.

Research results

After parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats, there is failed vasculature, and finally, increased adhesion formation. NO-agents, L-NAME and/or L-arginine have diverse effects, the initial weakening of blood vessel disappearance and finally, severe worsening of adhesions (L-NAME) *vs* the initial weakening of blood vessel disappearance and finally, attenuation of adhesions formation (L-arginine), which counteract each other response given together. The application of the stable gastric pentadecapeptide BPC 157 with its most recent described vascular effects ("vascular recruitment") recovers abundant vascular vessel presentation in and close to the defect, which occurs rapidly. Finally, BPC 157 attenuated bowel adhesion formation and NO- and MDA-tissue values.

Research conclusions

BPC 157 therapy can be suited for the realization of the peritoneal defect healing with minimal or no adhesion formation. In practical BPC 157 therapy realization, establishing vessels-adhesions-BPC 157-NO-system relation may be important. Finally, BPC 157, with LD1 not achieved, implemented in inflammatory bowel disease trials, with a particular effect on vessel presentation and counteracting effect on NO-agents harmful effect, free radical formation, and NO-values normalization, should be the practical hallmark of the further therapy to avoid adhesion formation.

Research perspectives

This study of abdominal wall injury has basic relationships, such as more vascular failure (vessels, since empty "disappear") at a very early post-injury period, as the more failed outcome (*i.e.*, the huge adhesion formation). MDA values and NO-levels increased at the initial abdominal wall defect, and thus increase during subsequent adhesions. On the contrary, the possible recovering effect is that the filled vessels "reappear"; adhesion formation attenuated. Decreasing of the increased NO- and MDA- values match with the beneficial outcome (less adhesion formation). Only the BPC 157 therapy in the damaged abdominal wall healing fulfils this combined beneficial effect. The very rapid activation of the blood vessels in the initial defect likely circumvents subsequent negative chain of events and results in the resolution of the adhesion and obstruction, decreasing of the increased NO- and MDA values match with the beneficial outcome (less adhesion formation).

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ORIGINAL ARTICLE

Retrospective Study

Do liver metastases from gastric cancer contraindicate aggressive surgical resection? A 14-year single-center experience

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Abstract

BACKGROUND

Advanced gastric cancer (GC) with liver metastasis is often characterized by multiple and bilobular metastases and may also be associated with extrahepatic metastatic lesions. Hence, many physicians consider that radical surgeries are contraindicated for liver metastases from GC (LMGC). According to the 2017 Japanese treatment guideline for GC, a smaller number of liver metastases without unresectable factors may be an indication for liver resection (LR) with curability. The actual 5-year overall survival (OS) rate ranges from 0 to 0.37.

AIM

To present the institutional indications for LR for LMGC and identify important factors for prognostic outcomes.

METHODS

In total, 30 patients underwent LR for LMGC during a 14-year period, and we evaluated the clinical, surgical, and oncological findings. In all patients, radical surgery with intentional lymphadenectomy was performed for the primary GC. The median follow-up duration after the initial LR was 33.7 mo, and three patients with no recurrence died of causes unrelated to the LMGC. The OS and recurrence-free survival rates after the initial LR were assessed.

RESULTS

Seventeen patients had metachronous LMGC. The initial LR achieved curability in 29 patients. Perioperative chemotherapy was introduced in 23 patients. The median greatest LMGC dimension was 30 mm, and the median number of LMGC

authorizing the use and disclosure of their protected health information.

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was two. Twenty-two patients had unilobular LMGC. The 5-year OS and recurrence-free survival rates were 0.48 and 0.28, respectively. The median survival duration and recurrence-free duration after the initial LR were 16.8 and 8.6 mo, respectively. Twenty-one patients developed recurrence after the initial LR. Additional surgeries for recurrence were performed in nine patients, and these surgeries clearly prolonged the patients' survival. Pathological serosal invasion was an independent predictor of a poor prognostic outcome after the initial LR. Aggressive LR may be indicated for carefully selected patients with LMGC.

CONCLUSION
Our results of LR for LMGC seem acceptable. Additional surgeries for recurrence after the initial LR might prolong OS. Pathological serosal invasion is important for poor prognostic outcomes.

Key Words: Liver metastasis; Metastatic tumor; Gastric cancer; Hepatectomy; Liver resection; Surgery

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Core Tip: The indication for liver resection (LR) for liver metastases from gastric cancer (LMGC) is still controversial. In the present study, the institutional indications for LR for LMGC are shown in detail. The 5-year overall survival rate was 0.48, and our results of LR for LMGC seemed to be acceptable. Although recurrence might develop after the initial LR, additional surgeries for recurrence clearly prolong survival. Pathological serosal invasion of the primary gastric cancer was an independent predictor of poor prognostic outcomes after the initial LR. Aggressive LR may be indicated for carefully selected patients with LMGC.

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INTRODUCTION

Advanced gastric cancer (GC) with liver metastasis is often characterized by multiple and bilobular metastases and may be associated with extrahepatic metastatic lesions. Hence, many physicians consider that radical surgery is contraindicated for these patients. According to the Japanese treatment guideline for GC[1], a smaller number of liver metastases without unresectable factors (*i.e.*, extrahepatic metastases) may be an indication for radical surgery if strict curability (*i.e.*, graphical and surgical R0, according to the Japanese classification of gastric carcinoma[2]) will be accomplished.

The evidence regarding the indications for liver resection (LR) for liver metastases from GC (LMGC) is limited, and much of the research on this topic consists of single-center retrospective studies with a small sample size[1,3]. These studies suggested that the surgical curability of LR and a smaller number of LMGC were important factors for prognostic outcomes after LR[3] and that aggressive LR for LMGC may prolong survival in carefully selected patients who undergo radical surgery for the primary GC[3]. A later study showed that a solitary metastasis was an important independent factor for prognostic outcomes after LR[4]. Three or fewer LMGC in highly accurate imaging studies is an indication for LR, and the 5-year overall survival (OS) rate is approximately 0.3 in this population[1,5,6]. The effectiveness of LR for patients with synchronous or metachronous LMGC is still controversial[3]; therefore, any patients with synchronous or metachronous LMGC may be candidates for LR[3]. Several factors have been clearly detected as important independent predictive factors for postoperative recurrence and/or prognostic outcomes after LR[4-19], and the actual 5-

year OS rate reportedly ranges from 0 to 0.37[5-19].

As described above, the surgical approach may have therapeutic potential for carefully selected patients with LMGC[3]. We have aggressively recommended LR for these patients. In the present study, we retrospectively evaluated our patients with LMGC who underwent both a radical surgery for the primary GC and LR for LMGC during the past two decades. We present our institutional indications and contraindications for LR in patients with LMGC. From the viewpoint of clinical oncology, the goal of this study was to identify the most important predictive factors for postoperative recurrence and/or prognostic outcomes after LR.

MATERIALS AND METHODS

Evaluation and assessment of oncologic findings, clinical therapies, and actual responses

In this study, the oncologic findings were evaluated and reported according to the Japanese classification of gastric carcinoma [*i.e.*, tumor depth (T factor), cytology (CY factor), pathological lymphatic invasion (ly factor), and pathological vessel invasion (v factor)][2]. Additionally, clinical therapies and actual responses were assessed and described according to the Japanese treatment guideline for GC[1] [*i.e.*, intentional lymphadenectomy (D number), graphical or surgical curability (R number), and clinical response after chemotherapy].

Institutional indication for LR for LMGC

The indications and contraindications for LR for LMGC at our institution are summarized in Table 1. There were no restrictions in the distribution of metastases (unilobular or bilobular), cytology (CY0 or CY1), or timing of metastasis (synchronous or metachronous).

The indications for LR were as follows: (1) The primary GC could be curatively resected by radical gastrectomy with intentional lymphadenectomy; (2) All LMGC could be resected with no metastasis in the remnant liver; (3) The volume of the remnant liver was graphically estimated as > 40% against the whole liver; (4) The number of LMGC was ≤ 5, though the size of the LMGC was not limited; and (5) No unresectable site was observed. In brief, the curability of LR (*i.e.*, graphical and surgical R0[2]) is of utmost importance, and we accomplish curability whenever possible.

In contrast, the contraindications for LR were as follows: (1) Five or more metastases were present; (2) Peritoneal dissemination was observed; and (3) An extrahepatic unresectable site was present. In brief, the incurability of LR (graphical or surgical remnant of metastasis) was a strong contraindication for any type of hepatectomy.

Therapeutic strategy for LMGC in our institution

The therapeutic strategy for LMGC at our institution is summarized in Figure 1. If LMGC were resectable, LR was initially performed and adjuvant chemotherapy was subsequently introduced. If LMGC were resectable but involved some difficulties for LR (*e.g.*, higher number and larger size of liver metastases), chemotherapy was initially introduced as neoadjuvant chemotherapy (NAC) and LR was performed thereafter only in patients with a positive response (*i.e.*, complete response, partial response, or stable disease according to the Japanese classification of gastric carcinoma[2]). If LMGC were unresectable, systemic chemotherapy was introduced. LR was performed as conversion surgery only in patients who fulfilled our institutional indications.

Patients

During the 14-year period from January 2006 to January 2020, a total of 1086 patients with GC underwent surgical treatment in our institution. Thirty patients who underwent LR for LMGC were enrolled in this study. We retrospectively evaluated our own results after LR (*i.e.*, clinical course, pathological findings, postoperative recurrence, and prognostic outcomes).

In all cases, thoracoabdominal enhanced computed tomography were routinely performed for checking extrahepatic diseases. Positron emission tomography/computed tomography was also used.

Ethical approval

This retrospective study was approved by our institution's ethics review committee for

Table 1 Indication and contraindication for liver resection for liver metastases originated from gastric cancer.

Indicated	Contraindicated
Radical resection of the primary GC	Tumor number of > 5
No tumor in the remnant liver (curability of LR)	Peritoneal dissemination (incurability of LR)
Remnant liver volume of > 40%	Extrahepatic unresectable site (incurability of LR)
Tumor number of ≤ 5	
Any tumor size	
No unresectable site (curability of LR)	
Regardless of unilobular or bilobular LMGC	
Regardless of cytology (CY0 or CY1)	
Regardless of synchronous or metachronous LMGC	

GC: Gastric cancer; LR: Liver resection; LMGC: Liver metastases originated from gastric cancer.

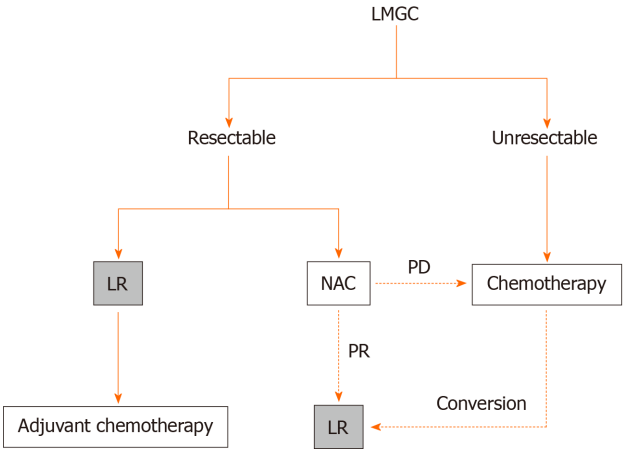


Figure 1 Therapeutic strategy for liver metastases originated from gastric cancer. Japanese classification of gastric carcinoma: definitions of PD and PR. LMGC: Liver metastases originated from gastric cancer; LR: Liver response; NAC: Neoadjuvant chemotherapy; PR: Partial response; PD: Progressive disease.

clinical studies. The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients before enrollment.

Statistical analysis

All results are shown as mean ± standard deviation or median (range). Survival rates were calculated using the Kaplan–Meier method, and the log-rank test was used for between-group comparisons. The Mann–Whitney *U* test and χ^2 test were used to compare unpaired continuous or discontinuous variables between two groups. Logistic regression analysis was used to identify important predictive factors for poor prognostic outcomes. The effect of each factor on OS was evaluated by multivariate cyclooxygenase regression analysis. All calculations were performed using SPSS software (SPSS Inc., Chicago, IL, United States). A P value of < 0.05 was considered statistically significant.

RESULTS

Clinical and pathological findings of primary GC

This study included 23 men and 7 women. Their GC was localized at the corpus (M portion) (n = 13), the antrum and pylorus (L portion) (n = 10), and the fundus (U portion) (n = 7)[2]. The greatest dimension was 63.3 ± 29.2 mm, and the primary GC pathologically disappeared after NAC in one patient (*i.e.*, complete response).

Pathologically, 22 patients had well/moderately differentiated tubular adenocarcinoma (tub1/tub2); 4 had poorly differentiated adenocarcinoma (por); and 1 each had papillary adenocarcinoma (pap), mucinous adenocarcinoma (muc), endocrine cell carcinoma, and hepatoid adenocarcinoma. No signet-ring cell carcinoma (sig) was observed. One differentiated adenocarcinoma secreted alpha-fetoprotein.

In the pathological assessments, the tumor depth (T factor) was evaluated as T3 (ss) (n = 16), T4b (si) (n = 6), T4a (se) (n = 5), and T1b (sm) (n = 2). No tumor was observed in the one patient with a complete response after NAC. Vessel invasion (positive v factor) was observed in 28 patients. Twenty-one patients had lymphoid invasions (positive ly factor), and 25 patients had metastatic lymph nodes (LNs) (positive N factor). Cytology (CY factor) was positive in one patient, and peritoneal dissemination (P1[2]) was observed in two patients.

Surgical treatment of primary GC

Radical surgery with intentional lymphadenectomy (D2 or D2+[1]) was performed for the primary GC. Total gastrectomy, distal gastrectomy, and pancreaticoduodenectomy was performed in 16, 13, and 1 patient, respectively. The stage of the primary GC at the time of surgery was IV in 16 patients, IIIA in 6 patients, IIB in 3 patients, IIIC in 3 patients, IB in 1 patient, and IIA in 1 patient.

Timing of LMGC

Radical surgeries including gastrectomy and lymphadenectomy were performed for the primary GC in all patients. Seventeen patients had metachronous LMGC and 13 had synchronous LMGC (Figure 2).

NAC, LR, and adjuvant chemotherapy

Seven patients with metachronous LMGC underwent LR, and two of these seven patients subsequently received adjuvant chemotherapy (uracil and tegafur or S-1). Four patients with LMGC received chemotherapy [S-1, trastuzumab, ramucirumab, S-1 + paclitaxel, S-1 + cisplatin (CDDP), irinotecan (CPT-11) + CDDP, or capecitabine + oxaliplatin], and LR was performed thereafter. The response to NAC was a partial response in two patients and stable disease in two patients. Four of these 10 patients received adjuvant chemotherapy (S-1 or CPT-11 + CDDP). Six patients received no chemotherapy (Figure 2).

Ten patients with synchronous LMGC underwent LR, and 9 of these 10 patients thereafter received adjuvant chemotherapy (S-1). Three patients with synchronous LMGC received chemotherapy (S-1 + oxaliplatin, S-1 + paclitaxel, or S-1 + CDDP), and LR was performed thereafter. All patients had a partial response to NAC. None of these three patients received adjuvant chemotherapy. One patient with synchronous LMGC received no chemotherapy (Figure 2).

Surgical procedures and curability of LR

The mean age at the time of LR was 67.4 ± 8.4 years. Partial hepatectomy was performed in 16 patients, and the other 14 patients underwent major hepatectomy (*e.g.*, lobectomy and segmentectomy). The major hepatectomies were left lobectomy (n = 5), right lobectomy (n = 2), extended right lobectomy (n = 1), right lobectomy + partial hepatectomy (n = 1), right anterior segmentectomy + lateral segmentectomy (n = 1), right posterior segmentectomy + partial hepatectomy (n = 1), extended left hepatectomy (n = 1), left hepatectomy + partial hepatectomy (n = 1), and lateral segmentectomy + partial hepatectomy (n = 1).

Curative LR (graphical and surgical R0[2]) was achieved in 29 patients. Curative LR was not achieved in one patient because of bone metastasis, but postoperative radiation therapy was performed in this patient.

Characteristics of LMGC

The size and number of LMGC were graphically and macroscopically assessed. The median greatest dimension of LMGC was 30 mm (range, 5-120 mm), and seven patients had a tumor size of > 5 cm. The median number of LMGC was 2 (range, 1-6), and one patient had > 5 LMGC postoperatively. Seventeen patients had multiple LMGC, and 13 patients had a solitary liver metastasis. Twenty-two patients had unilobular LMGC and eight had bilobular LMGC.

Survival after initial LR

The median follow-up duration was 33.7 mo [range, 0.8 (death of unrelated causes) to 157.1 mo]. Three patients with no recurrence after the initial LR died of unrelated

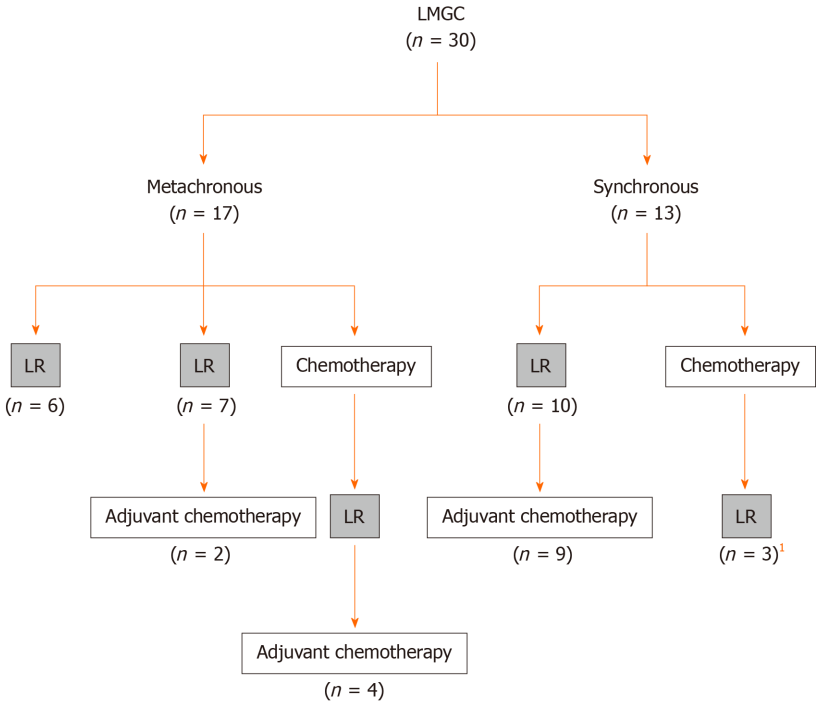


Figure 2 Actual treatments for synchronous and metachronous liver metastases originated from gastric cancer. Seventeen patients had metachronous liver metastases originated from gastric cancer (LMGC) and 13 patients had synchronous LMGC. Seven patients with metachronous LMGC underwent liver resection (LR), and two of these seven patients subsequently received adjuvant chemotherapy. Four patients with metachronous LMGC received chemotherapy, and LR was subsequently performed. A partial response to neoadjuvant chemotherapy was achieve in two patients. Four of these 10 patients received adjuvant chemotherapy. Ten patients with synchronous LMGC underwent LR, and 9 of these 10 patients subsequently received adjuvant chemotherapy. Three patients with synchronous LMGC received chemotherapy, and LR was subsequently performed. All responses to neoadjuvant chemotherapy were categorized as partial responses. [1]One case of conversion was involved. LMGC: Liver metastases originated from gastric cancer; LR: Liver resection.

causes during follow-up.

The actual OS curve after the initial LR is shown in Figure 3A. The 1-, 2-, 3-, and 5-year OS rate was 0.78, 0.63, 0.48, and 0.48, respectively. Among the 15 patients who died of oncological causes related to their GC (excluding 3 patients who died of unrelated causes), the median survival period after the initial LR was 16.8 mo (range, 3.7–84.0 mo).

The actual recurrence-free survival curve after the initial LR is shown in Figure 3B. The 1-, 2-, 3-, and 5-year recurrence-free survival rate was 0.49, 0.28, 0.28, and 0.28, respectively. Among the 21 patients with recurrence, the median recurrence-free period after the initial LR was 8.6 mo (range, 1.2-18.0 mo).

Postoperative recurrence after initial LR and additional surgeries

Although no recurrence was observed in 9 patients (30.0%), recurrence appeared after the initial LR in 21 patients (70.0%). When these 21 patients with recurrence were asymptomatic and ambulant, the target sites of recurrence after the initial LR were the liver (12 patients, 40.0%), LNs (8 patients, 26.7%), lung (4 patients, 13.3%), and peritoneum (2 patients, 6.7%). The target sites of the first recurrence after the initial LR were the liver (n = 8), LNs (n = 5), lung (n = 2), liver + lung (n = 2), liver + LNs (n = 2), peritoneum (n = 1), and liver + LNs + peritoneum (n = 1) (Table 2).

Additional surgeries were performed in nine patients with recurrence after the initial LR. These additional surgeries were LR (n = 3), LN dissection (n = 2), lung resection (n = 2), LR + LN dissection (n = 1), and LR + lung resection (n = 1). A total of eight LRs were repeated in five patients with liver recurrence (Table 2). Additionally, four lung resections were repeated in three patients with lung recurrence. One patient received additional LR, and moreover, this patient underwent lung resections twice (Table 2).

Each additional surgery achieved curability (graphical and surgical R0). Among the 21 patients with recurrence, the 9 patients who underwent additional surgeries for recurrence showed significantly longer survival than the 12 patients who did not undergo additional surgeries (P = 0.0014) (Figure 4).

Table 2 Postoperative recurrence after the initial liver resection and additional surgery

Postoperative recurrence after the initial LR and additional surgery	*n*
The target sites at the first recurrences after the initial LR (*n* = 21)	
Liver	8
LNs	5
Liver and LNs	3
Lung	2
Liver and lung	2
Liver, LNs and peritoneum	1
Peritoneum	1
Additional surgeries for postoperative recurrences after the initial LR (*n* = 9)	
LR	3
LN dissection	2
Lung resection	2
LR and LN dissection	1
LR and lung resection	1

LR: Liver resection; LN: Lymph node.

Important predictive factors for prognostic outcome after initial LR

Previously reported important predictive factors for postoperative recurrence and/or the prognostic outcome after the initial LR[4-19] were analyzed in the present study and are shown in Table 3. In the univariate analysis, serosal invasion (*i.e.*, pathological T factor) was significantly different from the other factors (P = 0.0249). The multivariate analysis showed that this factor was an independent predictor of a poor prognostic outcome after the initial LR (P = 0.0249).

No predictors of postoperative recurrence after initial LR

Previously reported important factors for postoperative recurrence and/or the prognostic outcome after the initial LR[4-19] were analyzed in the present study and are shown in Table 4. In the univariate analysis, no factors showed statistical significance. We found no predictive factors for postoperative recurrence after the initial LR.

DISCUSSION

Optimal surgeries for GC have been previously investigated. Intentional dissection of regional LNs (D2 Lymphadenectomy) is currently considered to be the standard lymphadenectomy technique for advanced GC[1]. Extended lymphadenectomy of para-aortic LNs does not improve the postoperative survival rate[20], and splenectomy for extended lymphadenectomy should be carefully chosen[21]. NAC before radical surgery is recommended for treatment of advanced but resectable GC, especially in patients with massive metastases in regional LNs[22,23].

The therapeutic strategy for stage IV GC has been previously discussed[24-32]. Improved prognostic outcomes have not been reported for surgeries that result in incurability (*i.e.*, R1 or R2[2]), and these surgeries are currently contraindicated for patients with GC at unresectable sites[24,27]. The surgical curability is crucial for patients with stage IV GC, and adjuvant chemotherapy after curative surgery (*i.e.*, graphical and surgical R0) is strongly recommended in this patient population[1]. In our two patients with peritoneal dissemination, there were no distant disseminations. Their disseminations were localized nearly at the primary GC (*i.e.*, P1[2]), and graphical and surgical R0 was accomplished by peritonectomy in each patient.

Paradoxically, aggressive surgeries that achieve curability (*i.e.*, graphical and surgical R0) may have potential benefits for patients with stage IV GC[5,8,12,33]. The surgical techniques of LR are well established, and medical devices for LR have been

Table 3 Important factor for prognostic outcome after the initial liver resection

	Factor	*P* value
Univariate analyses		
Number of LMGC	Actual number	0.7670
	Multiple *vs* solitary	0.8215
Timing of LMGC	Metachronous *vs* synchronous	0.3282
Occupation of LMGC	Bilobular *vs* unilobular	0.8605
The greatest dimension	Actual dimension (mm)	0.6264
	Size of > 50 mm *vs* size of ≤ 50 mm	0.2520
Serosal invasion (pathological T factor)	PT4 *vs* pT1-3	0.0249
Curability of LR	Yes or no	0.9999
Lymphatic invasion (pathological ly factor)	Yes or no	0.8004
Vessel invasion (athological v factor)	Yes or no	0.9999
Pathological differentiation	Tub *vs* others	0.8004
Pathological LN metastases	Yes or no	0.6171
Chemotherapy	Yes or no	0.4017
Multivariate analyses		
Serosal invasion (pathological T factor)	PT4 *vs* pT1-3	0.0052

LMGC: Liver metastases originated from gastric cancer; LR: Liver resection; LN: Lymph node.

Table 4 Univariate analyses for postoperative recurrence after the initial liver resection

	Factor	*P* value
Number of LMGC	Actual number	0.7860
	Multiple *vs* solitary	0.9360
Timing of LMGC	Metachronous *vs* synchronous	0.0906
Occupation of LMGC	Bilobular *vs* unilobular	0.5719
The greatest dimension	Actual dimension (mm)	0.7343
	Size of > 50 mm *vs* size of ≤ 50 mm	0.5719
Serosal invasion (pathological T factor)	PT4 *vs* pT1-3	0.8033
Curability of LR	Yes or no	0.9999
Lymphatic invasion (pathological ly factor)	Yes or no	0.9282
Vessel invasion (pathological v factor)	Yes or no	0.9999
Pathological differentiation	Tub *vs* others	0.9282
Pathological LN metastases	Yes or no	0.9999
Chemotherapy	Yes or no	0.9999

LMGC: Liver metastases originated from gastric cancer; LR: Liver resection; LN: Lymph node.

well developed. Hence, major or extended hepatectomy is a safe and feasible treatment option for liver disease[34]. Aggressive LR for LMGC may prolonged survival in carefully selected patients[4-19]. From the viewpoint of preoperative prediction of postoperative recurrence and/or poor prognostic outcomes, many surgeons have focused on the most important surgical indications for LR in patients with LMGC (Table 5): The timing of LMGC[6,13,15,17,18], the greatest dimension of LMGC[5,8-11], the multiplicity of LMGC[10,12,15-17], the occupation of LMGC[11-14], the number of LMGC[4,5,8],

Table 5 Important factors for liver resection in patients with liver metastases originated from gastric cancer

Ref.	Year	Sample size	The 5-yr OS rate	Important factors for recurrences and/or poor prognoses
Ambiru *et al*[18]	2001	40	0.180	Synchronous LMGC
Okano *et al*[17]	2002	19	0.340	Multiple LMGC; synchronous LMGC; pathological differentiation
Zacherl *et al*[12]	2002	15	0	Multiple LMGC; bilobular LMGC; curability of LR
Saiura *et al*[19]	2002	10	0.200	LN metastases
Shirab *et al*[8]	2003	36	0.260	Number of LMGC (number of ≥ 3); curability of LR; lymphatic invasion; vessel invasion
Sasako *et al*[20]	2007	37	0.110	Bilobular LMGC; the greatest dimension (size of > 4 cm)
Cheon *et al*[16]	2008	41	0.208	Multiple LMGC
Makino *et al*[14]	2010	16	0.370	Bilobular LMGC
Tsujimoto *et al*[10]	2010	17	0.315	Multiple LMGC; the greatest dimension (size of > 6 cm); lymphatic invasion
Schildberg *et al*[15]	2012	31	0.130	Multiple LMGC; synchronous LMGC
Takemura *et al*[9]	2012	64	0.370	The greatest dimension (size of > 5 cm); serosal invasion
Matsuda *et al*[13]	2013	14	0.360	Synchronous LMGC; bilobular LMGC; no chemotherapy
Kinoshita *et al*[5]	2015	256	0.313	The greatest dimension (size of > 5 cm); serosal invasion; curability of LR; number of LMGC (number of ≥ 3)
Tatsubayashi *et al*[6]	2017	28	0.320	Synchronous LMGC
Our study	2020	30	0.480	Serosal invasion (pathological T factor)

OS: Overall survival; LMGC: Liver metastases originated from gastric cancer; LN: Lymph node; LR: Liver resection.

the curability of LR[5,8,12], serosal invasion[5,9], lymphatic invasion[8,10], vessel invasion[8], LN metastases[19], induction of chemotherapy[13], and pathological differentiation[17]. Reliable reports published during the past two decades are summarized in Table 5.

The reported 5-year OS rate ranges from 0 to 0.37[5-19], and the rate in the present study was 0.48 (Table 5). Although the effectiveness of LR for LMGC remains controversial, the present results suggest that the OS rate after LR seems to be acceptable and that aggressive LR may be indicated in carefully selected patients with LMGC.

The curability of LR is important for patients with LMGC. In the present study, the initial LR was curative in 29 patients. Adjuvant chemotherapy after curative surgery (*i.e.*, graphical and surgical R0) is strongly recommended in patients with stage IV GC[28-32], and perioperative chemotherapy was introduced in 23 patients. GC patient with synchronous LMGC is categorized as Stage IV, and neoadjuvant and/or adjuvant chemotherapy will be introduced for synchronous LMGC as possible. Although neoadjuvant and/or adjuvant therapies were introduced in all of 13 patients with synchronous LMGC, 6 of 17 patients with metachronous LMGC did not receive perioperative chemotherapy (Figure 2). In these 6 patients with metachronous LMGC, the surgical curability (*i.e.*, graphical and surgical R0) of LR was accomplished in each. Though introduction of perioperative chemotherapy may involve a difficulty due to some reasons (*e.g.*, underlying disorder and performance status), and LR with surgical curability may be beneficial for metachronous LMGC patients who had some difficulty of perioperative chemotherapy. Recurrence after the initial LR occurred in 70% of patients. Notably, however, our results indicate that repeated additional surgeries for recurrence after the initial LR may be beneficial if surgical curability (*i.e.*, graphical and surgical R0) can be obtained (Figure 4). The present results of LR for LMGC may be supported by the combined strategy of aggressive curative surgeries and chemotherapy during the perioperative period of LR. Three of 6 patients with metachronous LMGC who did not receive perioperative chemotherapy were undergone additional surgeries for recurrence after the initial LR, and two of these 6 patients were still alive. Surgical curability is important for LR and additional surgery. LR and additional surgery may be beneficial for these metachronous LMGC patients if surgical curability is obtained, though we believe the combined strategy of aggressive curative surgeries and chemotherapy during the perioperative period of LR comes first

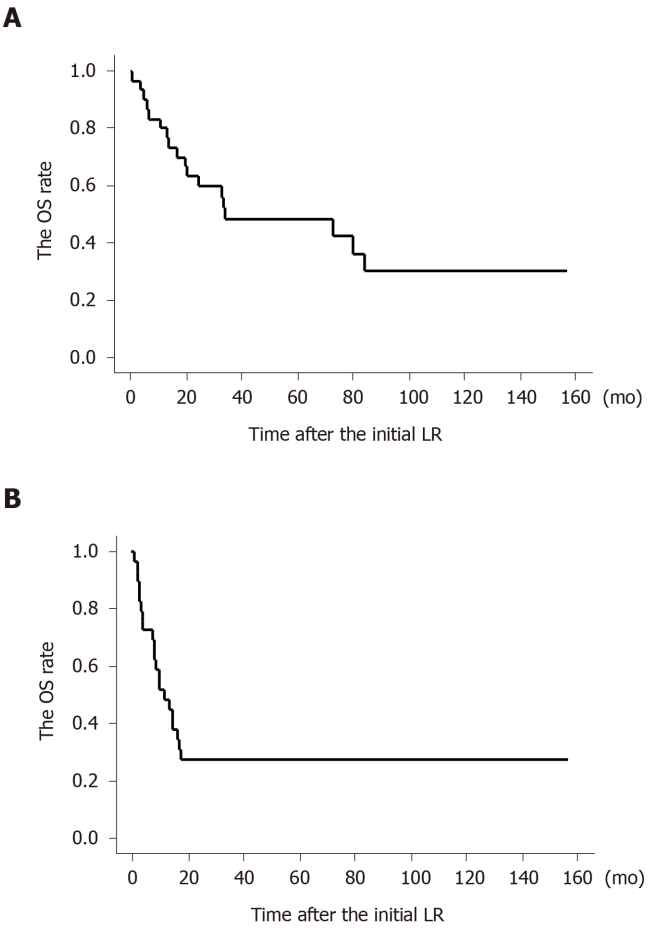


Figure 3 Overall survival and recurrence-free survival after initial liver resection. A and B: Actual curves of overall survival and recurrence-free survival. LR: Liver resection; OS: Overall survival; RFS: Recurrence-free survival.

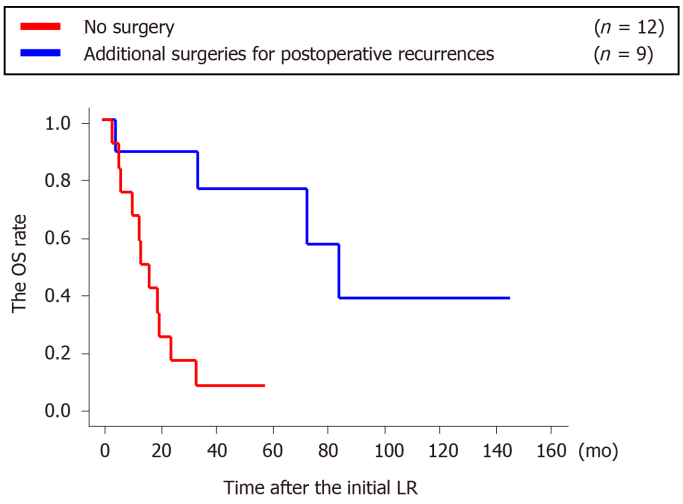


Figure 4 Survival curves in patients with recurrence. Recurrence after the initial liver resection was observed in 21 patients. Additional surgeries were performed in nine patients with recurrence, and each additional surgery accomplished graphical and surgical R0. These 9 patients had significantly longer survival than the other 12 patients who did not undergo additional surgeries ($P < 0.05$). LR: Liver resection; OS: Overall survival.

for patients with LMGC.

As shown in Table 5, only 14 papers have been previously documented, and almost all of these important papers were written based on retrospective design and/or single-center experience. Sample size were shown in Table 5. This was a retrospective study performed in a single institution and therefore has inherent limitations due to bias and a small sample size. Thus, the conclusions must be interpreted with extreme

caution. In 2018, when our sample size was smaller and follow-up term was shorter, the greatest dimension of > 50 mm was an independent predictor of postoperative recurrence after the initial LR in the univariate and multivariate analyses (data not shown). We found no important factors for postoperative recurrence after the initial LR (Table 4), although pathological serosal invasion was still an independent predictor of poor prognostic outcomes after the initial LR (Table 3).

Although resectable LMGC is clinically rare, strict and careful patient selection can lead to prolonged survival in patients with advanced GC. The surgical approach may have a therapeutic potential for LMGC.

CONCLUSION

In conclusion, aggressive LR may be indicated for carefully selected patients with LMGC, and the survival rate after LR seems to be acceptable.

ARTICLE HIGHLIGHTS

Research background

Advanced gastric cancer (GC) often accompanies with liver metastasis. Though many physicians consider that radical surgeries are contraindicated for liver metastases from GC (LMGC). a smaller number of liver metastases without unresectable factors may be an indication for liver resection (LR).

Research motivation

The actual 5-year overall survival (OS) rate was previously documented as 0 to 0.37. Here, we presented the institutional indications for LR for LMGC, evaluated our own results.

Research objectives

In total, 30 patients underwent LR for LMGC during a 14-year period, and we evaluated the clinical, surgical, and oncological findings.

Research methods

In all patients, radical surgery with intentional lymphadenectomy was performed for the primary GC. The median follow-up duration after the initial LR was 33.7 mo. The OS and recurrence-free survival rates after the initial LR were assessed. Also, we identified important factors for prognostic outcomes.

Research results

The 5-year OS and recurrence-free survival rates were 0.48 and 0.28, respectively. The median survival duration and recurrence-free duration after the initial LR were 16.8 and 8.6 mo, respectively. Although recurrence might develop after the initial LR, additional surgeries for recurrence clearly prolong survival. Pathological serosal invasion was an independent predictor of a poor prognostic outcome after the initial LR.

Research conclusions

Our results of LR for LMGC seem acceptable. Pathological serosal invasion is important for poor prognostic outcomes.

Research perspectives

Aggressive LR may be indicated for carefully selected patients with LMGC.

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