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INDEXING/ABSTRACTING
The WJG is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 impact factor (IF) for WJG as 5.374; IF without journal self cites: 5.187; 5-year IF: 5.715; Journal Citation Indicator: 0.84; Ranking: 31 among 93 journals in gastroenterology and hepatology; and Quartile category: Q2. The WJG’s CiteScore for 2021 is 8.1 and Scopus CiteScore rank 2021: Gastroenterology is 18/149.

RESPONSIBLE EDITORS FOR THIS ISSUE
Production Editor: Ying-Yi Yuan; Production Department Director: Xiang Li; Editorial Office Director: Jia-Ru Fan.

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

PUBLICATION DATE
September 7, 2022

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ONLINE SUBMISSION
https://www.f6publishing.com

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E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com
Regulation of transforming growth factor-β signaling as a therapeutic approach to treating colorectal cancer

Jana Maslankova, Ivana Vecurkovska, Miroslava Rabajdova, Jana Katuchova, Milos Kicka, Michala Gayova, Vladimir Katuch

Abstract

According to data from 2020, Slovakia has long been among the top five countries with the highest incidence rate of colorectal cancer (CRC) worldwide, and the rate is continuing to rise every year. In approximately 80% of CRC cases, allelic loss (loss of heterozygosity, LOH) occurs in the long arm of chromosome 18q. The most important genes that can be silenced by 18q LOH or mutations are small mothers against decapentaplegic homolog (SMAD) 2 and SMAD4, which are intracellular mediators of transforming growth factor (TGF)-β superfamily signals. TGF-β plays an important role in the pro-oncogenic processes, including such properties as invasion, epithelial-mesenchymal transition (commonly known as EMT), promotion of angiogenesis, and immunomodulatory effects. Several recent studies have reported that activation of TGF-β signaling is related to drug resistance in CRC. Because the mechanisms of drug resistance are different between patients in different stages of CRC, personalized treatment is more effective. Therefore, knowledge of the activation and inhibition of factors that affect the TGF-β signaling pathway is very important.

Key Words: Small mothers against decapentaplegic homologs; Transforming growth factor-beta; Colorectal cancer; Marker; Signaling pathway
Core Tip: A thorough understanding of the complete transforming growth factor (TGF)-β/small mothers against decapentaplegic homolog signaling pathway is important for defining its functions during pathological processes of colorectal cancer. Inhibitors specifically targeting TGF-β pathway mediators that reduce the expression of a particular protein may lead to fewer/milder adverse effects. However, the dual role of the TGF-β pathway in the onset and progression of cancer complicates the physiological/pathological and, thus, clinical situation. In recent years, research has shown that modification of members of this pathway is a promising approach for clinical procedures. Long-term treatment should emphasize personalized and targeted therapy.

Citation: Maslankova J, Vecurkovska I, Rabajdova M, Katuchova J, Kicka M, Gayova M, Katuch V. Regulation of transforming growth factor-β signaling as a therapeutic approach to treating colorectal cancer. World J Gastroenterol 2022; 28(33): 4744-4761
URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4744.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4744

INTRODUCTION

According to data from 2020, Slovakia has long been among the top five countries with the highest incidence rate of colorectal cancer (CRC) worldwide, and this rate continues to rise every year[1]. Although significant progress has been made in the diagnosis, screening, and treatment of patients with advanced CRC, therapeutic options are still limited, requiring the discovery of additional markers to act as prognostic predictors[2].

Up to 60%-65% of colorectal tumors have no family history (sporadic) and are the result of somatic mutations and epigenetic changes due to factors such as a lifestyle with limited physical activity, alcoholism and smoking[3]. CRC can arise as a result of these genetic and epigenetic aberrations (Figure 1): Chromosomal instability (CIN; 65%-85%), methylation of the CpG island (CIMP; 10%-20%), and DNA microsatellite instability (MSI; 12%-15%)[4]. Some authors have noted that patients with a tumor-bearing the CpG island methylator phenotype will have a worse prognosis compared to patients with a CIMP-negative tumor[5-7]. The instability of DNA microsatellite regions is characterized by mutations in the genome that arise due to defects in mismatch repair genes and can affect and inactivate tumor suppressor genes, leading to malignant transformation[8,9]. CIN is caused by the gain or loss of whole or large parts of chromosomes, leading to karyotype variability between cells. CIN results in chromosome imbalance (aneuploidy), subchromosomal genomic amplification, and loss of heterozygosity (LOH)[10].

This type of classification, based on a single molecular marker, is not very informative in the early diagnosis of CRC; thus, a combination of several molecular markers has been proposed as a better classification approach for patients with CRC. Moreover, the joint efforts of the CRC Subtyping Consortium have led to a formal proposal for the stratification of CRC cases into the following four molecular subtypes (referred to as CMS1-4)[11,12] (Figure 2; Table 1).

CMS1 is usually a right-sided (proximal) tumor, commonly diagnosed in older age females, and is associated with worse survival after relapse. This subtype is characterized by hypermethylation of CpG islands, which causes loss of tumor suppressor function and has a low prevalence of somatic copy number alterations (referred to as SCNs). The hypermethylation of promoter regions of the MMR genes causes MSI[11].

CMS2 is mainly located on the left side (distal part of the colon) and is often diagnosed in men, with a better prognosis and a higher survival rate, even after relapse. This gene expression profile is characterized by low mutation rate. CMS2 also represents over-activation of epidermal growth factor (EGF)-related signaling pathways, with higher expression of the epidermal growth factor receptor (EGFR)[13]. Finally, Guinney et al[11] reported that CMS2 has more copy number gains in oncogenes and losses in tumor suppressor genes than the other CMSs.

CMS3 is another right-sided subtype and is the most frequently diagnosed in patients with evident metabolomics disease[13]. Although KRAS mutation is present in every CMS, it occurs more frequently in CMS3[11].

CMS4 tumors exhibit extremely low levels of hypermutation and are defined by an activated transforming growth factor (TGF)-β pathway and by epithelial-mesenchymal transition (EMT), making them generally more chemoresistant[13]. CMS4 tumors tend to be diagnosed at more advanced stages (III and IV); indeed, the poor prognosis of CMS4 (compared to the relatively favorable prognoses of CMS1 and CMS2) in non-metastatic disease have been demonstrated[11].
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<table>
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Table 1 Characteristics of individual colorectal cancer subtypes

SCNA: Somatic copy number alteration; CIN: Chromosomal instability; CIMP: Methylation of the CpG island; TGF: Transforming growth factor.

Figure 1 Representation of individual colorectal cancer subtypes.

Figure 2 Three genetic and epigenetic aberrations of colorectal cancer formation. LOH: Loss of heterozygosity; TGF: Transforming growth factor.

The basic characteristics of each CRC subtype, CMS1-4, are summarized in Table 1. Approximately 80% of colorectal tumors have loss of an allele in the long arm of chromosome 18q, followed by LOH on chromosome 17p (75%-80%), 8p (40%), 5q (30%), and finally 22q (20%-30%). Allelic loss in chromosome 18q has been reported in 70% of cases of primary CRC with late-stage adenomas and shows a strong correlation with poor prognosis [14]. Patients with 18q LOH have a particularly poor prognosis in stage II disease, leading to the conclusion that stage II adjuvant therapy is important for these patients[15].
There are many candidate tumor suppressor genes in 18q, including small mothers against decapentaplegic homolog (SMAD) 2, SMAD4, netrin receptor DCC (DCC), and Cdk5 and Abl enzyme substrate 1 (CABLES1) [16]. The most important genes that can be silenced by 18q LOH or mutations are SMAD2 and SMAD4, which are intracellular mediators of TGF-β superfamily signaling [17].

**TGF-β SUPERFAMILY SIGNALING**

TGF-β superfamily signaling is mainly divided into the following two subfamilies: TGF-β-activin-nodal and bone morphogenetic protein (BMP). The TGF-β ligand (comprised of the TGF-β1, -β2, and -β3 isoforms) is a multifunctional member of the cytokine family, playing an important role in such cellular responses as cell proliferation, differentiation, and pathological processes. TGF-β itself plays a key role in the processes of EMT and fibrosis [18].

The canonical (SMAD-dependent) TGF-β signaling pathway (Figure 3) utilizes serine/threonine kinase receptors (TGF-βRI/TGF-βRII) in the plasma membrane and phosphorylates their cytoplasmic effectors SMAD2 and SMAD3. TGF-βRII receptors differ from TGF-βRI by the presence of an N-terminal glycine/serine-rich (GS) domain, which regulates kinase activity and SMAD binding. TGF-βRII receptor phosphorylates serine and threonine residues within the GS domain of TGF-βRI, and activated TGF-βRI receptor phosphorylates the distal C-termini of SMAD2 and SMAD3. An anchor of SMAD receptor activation, a SMAD cofactor that directly interacts with SMAD2/3, is required to anchor SMAD2/3 proteins to the TGF-β receptor. After phosphorylation, SMAD2 and SMAD3 dimers form heteromeric complexes with SMAD4 and then translocate to the nucleus. They act as transcription factors, mediate the expression of various genes, and promote various biological functions in the tumor microenvironment, resulting in tumor suppression [19].

A conserved branch of the TGF-β superfamily involves BMP signaling. BMP canonical signaling is triggered upon the binding of soluble ligands to serine-threonine kinase receptors, BMPRI and BMPRII, in the plasma membrane. Activated BMP receptors stimulate various intracellular signaling pathways. This canonical pathway is characterized by phosphorylation of SMAD1/5/8, which subsequently forms a gene-regulatory complex with SMAD4. Alternative BMP signaling can occur via the non-canonical pathway and is due to the presence of multiple intracellular kinases (Figure 3) [20].

While TGF-β-induced extracellular matrix production promotes tumor development, the inhibitory response to TGF suppresses tumor formation. Thus, the level of TGF-β receptor activation can alter the outcome of TGF-β signaling from suppression to oncogenesis. The TGF-β/SMAD signaling pathway has a dual effect; during tumor initiation and early stages, it stops the cell cycle and triggers apoptosis and in later stages, it promotes tumorigenesis and increases tumor progression and invasiveness [21]. TGF-β signaling causes cell cycle arrest and death during tumor initiation, acting as a tumor suppressor. However, it has also been demonstrated to increase tumor cell proliferation, EMT, and stem-like activity during tumor progression, as well as fibrosis, inflammation, and angiogenesis [22-24].

**TGF-β AND ITS ROLE IN TUMOR SUPPRESSION**

TGF-β signaling regulates cell proliferation mainly by inhibiting cell cycle progression through a mechanism that arrests the cell in the G1 phase. In most epithelial, endothelial, and hematopoietic cells, this arrest occurs through the activation of cyclin-dependent kinase (CDK) inhibitors, such as p21CIP1 and p15INK4b. TGF-β signaling also inhibits c-Myc oncogene transcription as well as DNA-binding protein inhibitors (ID1-3) and nuclear factors, which play key roles in cell differentiation and progression from the G1 to S phase of the cell cycle [25].

The canonical TGF-β signaling pathway can induce apoptosis by modulating the expression of various members of the B-cell lymphoma 2 (Bcl-2) family such as death receptor fibroblast death-associated antigen (FAS), DNA damage-inducible (GADD) 45-β, and kinase associated with death (DAPK), which depends on the type of cells where the signaling takes place. It can also induce growth arrest and modulate caspases to induce intrinsic and extrinsic apoptosis [19].

**TGF-β AND ITS ROLE IN TUMOR PROMOTION**

In later stages of cancer, TGF-β may adversely promote tumor progression and metastasis [18]. The TGF-β signaling pathway activates the promoter activity of the translation inhibitory protein 4E-BP1 (regulator of eukaryotic translation initiation factor-4F (eIF4E) through SMAD4, thereby suppressing translation, cell growth and proliferation [26]. TGF-β also promotes the secretion of matrix metalloproteases (MMPs), mainly MMP-2 and MMP-9, and inhibits the activity of their tissue inhibitors (TIMPs) [27].
Figure 3 Transforming growth factor-beta superfamily signal transduction. TGF: Transforming growth factor; EMT: Epithelial-mesenchymal transition; ERK: Extracellular signal-regulated kinase; BMP: Bone morphogenetic protein; SMAD: Small mothers against decapentaplegic homolog.

Fibrotic processes are well known to play a key role in promoting malignancy, and TGF-β is one of the most prominent inducers of fibrotic processes. During fibrosis, abundant ECM components accumulate due to activated myofibroblasts. In tumor tissue, solidified stroma stimulates tumor cell proliferation, migration, and survival. Fibrosis plays a vital role in EMT regulation, promotes angiogenesis and hypoxia, and inhibits anti-tumor immunity. Ultimately, the degree of tissue fibrosis is related to tumor aggression and poor patient prognosis[28].

TGF-β collaborates closely with BMP during fibrosis, due to their structural similarity and shared signal transmission modality. Their role is to regulate fibrosis-causing processes, like EMT. The interaction of TGF-β and BMP to form a complex with SMAD4, together with SMAD7 which elicits an inhibitory effect, affects the balance between the activation of SMADs that are members of the TGF-β signaling pathway (SMAD2/3) and SMADs that are part of the BMP signaling pathway (SMAD1/5/8). Therefore, many studies report antagonistic roles of TGF-β and BMP[29,30]; according to them, BMP activity is antifibrotic. Fewer studies support the opposite trend. Specifically, Katsuno et al[31] determined that BMP signaling can promote TGF-β signaling through the activation of protein arginine N-methyltransferase (PRMT1), which methylates SMAD6/7. SMAD6/7, in turn, activates SMAD1/3/5, resulting in the promotion of EMT during fibrosis and the maintenance of the tumor cell phenotype in malignancies[29,31].

TGF-β/SMAD RECEPTORS

Each of the isoforms of TGF-β (-1, -2, -3) binds to serine/threonine kinases, which belong to the group of transmembrane receptors and can bind to TGF-β1 and TGF-βII. The name of TGF-βRI is also an activin-like receptor kinase (ALK). Seven types of TGF-βRIs have been identified to date (ALK1-7), five types of TGF-βRIIs (TGF-βRII, BMPRII, ACVRII, ACVRIIB, and AMHRII), and two types of TGF-βRIIIIs (betaglycan and endoglin). All TGF-βRIs consist of a C-terminal cytoplasmic domain of a serine/threonine kinase, an internal transmembrane region, and an N-terminal domain, which binds ligands [18].

TGF-β receptors, SMAD proteins, and their mutation or inactivation have been described in many publications, along with their role in the progression of malignancies[32,33]. The loss of TGF-β tumor suppressor functions, which play a key role in inhibition in normal epithelial cells as well as in tumor cells, leads to oncogenic processes. Many human cancers, including CRC, are resistant to TGF-β-mediated growth inhibition, however. This resistance may be due to mutation or functional inactivation.
of TGF-βRI, decreased expression of TGF-βRI or TGF-βRII, and inactivation mutations of individual members of the TGF-β signaling pathway, such as SMAD2 and SMAD4[25]. Reportedly, approximately 20%-30% of CRCs contain mutations of TGF-βRII, and mostly involve colon cancer cells with MSI. One of the most frequent MSI mutations detected occurs in a coding polyadenine tract in exon 3 of the TGF-βRII gene. Some studies have even suggested that one of the important factors contributing to CRC transformation is the inactivation of TGFβR2, which increases cell proliferation due to prolonged activation of cdk4 expression[34-36].

Not only TGF-βRII but also TGF-βRI may contain a similar hypermutable polyadenine sequence resulting from mismatch repair defects, and the mutant allele (known as TGF-βRII6A) has been described to predispose to colon cancer, with a reported frequency of 100%[37].

### SMADs

The mammalian TGF-β receptor family contains five SMAD substrates (SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8); these are commonly referred to as receptor-regulated SMADs or R-SMADs[19]. Bone morphogenetic protein (BMP) and anti-Müller receptors have high affinity for SMAD 1, 5, and 8 and TGF-β, activin and nodal receptors bind SMAD 2 and 3 proteins. SMAD4 belongs to the co-SMAD group, the second class of the SMAD family, which serves as a common partner for all R-SMADs such as SMAD2, SMAD3, SMAD1, SMAD5, and SMAD8 to form heterotrimeric complexes. These heterotrimeric SMAD complexes are subsequently translocated to nuclei, where they bind to specific promoters to act as DNA-specific transcriptional regulators of target genes[38]. SMAD6 and SMAD7 have inhibitory roles in the TGF-β/SMAD signaling pathway[39,40].

SMAD proteins are composed of approximately 500 amino acids and consist of two globular domains [MAD homology (MH) 1 and MH2] joined by a linker region. The N-terminal domain of MH1 is highly conserved in all R-SMADs and SMAD4, but not in SMADs 6 and 7, and contains a hairpin structure with DNA-binding capability. The MH2 domain contains hydrophobic elements that bind to TGF-βR and BMPR transmembrane receptors. The linker region is quite different between the different subgroups, whereas the C-terminal domain (MH2) is identical in all SMAD proteins[19]. The MH2 domain is involved in SMAD protein homooligomerization and heterooligomerization, cytoplasmic anchoring, and transcription. In normal (healthy) and premalignant cells, the TGF-β tumor signaling pathway has a suppressive role, but this pathway can be inhibited, damaged, or even used by cancer cells to promote oncogenic functions[38]. The known roles of individual SMAD proteins during the onset and progression of CRC are summarized in Table 2.

In 65% of colon adenocarcinomas and 50% of rectal adenocarcinomas, mutations in any of the 43 genes that encode proteins of the TGF-β pathway superfamily have been described[19]. Many proteins interact with the SMADs to modulate their activity. Therefore, by regulating these proteins, we can influence the process of carcinogenesis[41].

### Role of SMAD2/3

Many studies describe the significant role of SMAD2/3 in the EMT process, which is activated by the TGF-β signaling pathway. The most important difference between SMAD2 and SMAD3 is that the MH1 region of SMAD2 has two more amino acid fragments than SMAD3. Due to this amino acid difference, SMAD3 can directly bind to DNA and has transcriptional activity, whereas SMAD2 lacks transcriptional activity[42,43].

Although SMAD3 is highly homologous to SMAD2, the roles of SMAD2 and SMAD3 are different in the TGF-β signaling process. SMAD3 plays a very important role as a mediator of EMT, as demonstrated by inhibition or knockdown of SMAD3, which blocked cell migration induced by the TGF-β signaling pathway. Therefore, regulation of SMAD3 protein expression is a very important regulatory step in EMT prevention[44].

The results of Liu et al[45] pointed to other important differences between SMAD2 and SMAD3. SMAD2 is mostly located in the cytoplasm, whereas a large amount of SMAD3 is distributed in the nucleus. Western blot analysis was performed in that study, which provided evidence to support the conclusion that in the absence of TGF-β activation, endogenous SMAD2 is found mainly in the cytoplasm, while large amounts of SMAD3 are found in the nucleus of human embryonic stem cells, kidney cells, and skin fibroblast cells. This otherwise in different cell compartments of SMAD2 and SMAD3 proteins may reflect their activity in TGF-beta-induced signal transduction.

Analyses of tissue and experiments with explanted tissue have revealed strongly reduced phosphorylated SMAD3 and increased levels of its inhibitor SMAD7 in Crohn’s disease tissue and a moderate reduction in ulcerative colitis (UC) tissue[46]. UC poses a high risk of developing CRC; however, the molecular mechanisms underlying the transition from UC to CRC are unclear[47].

Wang et al[48] showed that it was possible to increase the transcriptional activity of SMAD3, phosphorylation of SMAD2, and reduction of SMAD7 expression by knocking out signal transducer and activator of transcription 3 (STAT3), which ultimately led to the suppression of tumor progression in CRC. STAT3 is a member of the STAT protein family and can promote oncogenesis of CRC through
Table 2 Roles of individual small mothers against decapentaplegic homolog proteins in the onset and progression of colorectal cancer

<table>
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<th>Type of SMAD</th>
<th>Role in colorectal cancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMAD1</td>
<td>Participates in the modification of cell growth, differentiation, apoptosis and other processes that are essential in the regulation of the body’s immune system. Promotes epithelial-mesenchymal transition process. By increasing the expression of ATG5 induces autophagy.</td>
<td>[39-42]</td>
</tr>
<tr>
<td>SMAD2</td>
<td>Inhibits the expression of related functional genes, cell proliferation and regulates the transcriptional response that promotes cell apoptosis. Expression of SMAD2 is correlated with patient survival.</td>
<td>[43,44]</td>
</tr>
<tr>
<td>SMAD3</td>
<td>In the formation of a tumor, depending on the stage of the cancer, it plays the double role of an oncogene or a tumor suppressor gene. Reduces its expression through mir-4429, and inhibits the appearance, development and metastasis of cancer cells.</td>
<td>[45-48]</td>
</tr>
<tr>
<td>SMAD4</td>
<td>Plays a very important role in the transduction of the TGF-β signaling pathway. Maintains the cell cycle in the G1 phase, which leads to abnormal tumor proliferation. Is a tumor suppressor gene. High mutation rate of SMAD4 in CRC patients was associated with poor prognosis, but not with clinical stage.</td>
<td>[32,49]</td>
</tr>
<tr>
<td>SMAD5</td>
<td>Mediates TGF-β superfamily ligand signaling pathway and thus influences cancer progression.</td>
<td>[50]</td>
</tr>
<tr>
<td>SMAD6</td>
<td>Regulates TGF-β signaling pathway, promotes angiogenesis, stimulates extracellular matrix, and inhibits immunity, thus contributing to tumor growth, diffusion, and metastasis.</td>
<td>[51]</td>
</tr>
<tr>
<td>SMAD7</td>
<td>Plays a dual role in different tumor stages, acting as a tumor suppressor gene by inhibiting proliferation and promoting apoptosis in the early stage, and increasing invasion in the late stage, promoting epithelial-mesenchymal transition, which correlates with the degree of malignancy</td>
<td>[52,53]</td>
</tr>
</tbody>
</table>

SMAD: Small mothers against decapentaplegic homolog; ATG5: Autophagy-related gene 5; CRC: Colorectal cancer; TGF: Transforming growth factor.

Various pathways.

Liu et al.\cite{49} reported that treatment with exogenous interleukin 6 (IL-6) stimulated STAT3 activation, increased TGF-β-induced SMAD3 and Snail expression, and inhibited the EMT process, suggesting that the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway is required for TGF-β-induced EMT and cancer cell migration and invasion by upregulating SMAD3 and Snail expression. Moreover, Xu et al.\cite{50} showed that the expression of SMAD2 is correlated with patient survival. Their results demonstrated that the MIR22 host gene (MIR22HG) has been shown to play a role in suppressing colorectal tumors by binding competitively to SMAD2, thereby preventing the interaction between SMAD2 and SMAD4. These data suggest that the MIR22HG silencing promotes the EMT process and thus tumorigenicity in CRC.

Many papers have been published in recent years that link the action of the TGF-β signaling pathway to other pathways. The mitogen-activated protein kinase (MAPK) pathway may phosphorylate a group of proteins that are responsible for altering cell behavior, or conversely, proteins of this pathway may be activated by extracellular molecules such as cytokines produced by the TGF-β signaling pathway. The extracellular signal-regulated kinase (ERK) pathway inhibits the TGF-β pathway by phosphorylating SMAD2 and SMAD3 without translocating them to the nucleus\cite{51,52}.

Despite the important roles of SMAD2 and SMAD3 in the TGF-β signaling process, the prevalence of mutations was estimated up to 6%. Fleming et al.\cite{53} showed that the percentage of mutations increased with the combined prevalence of SMAD4, SMAD2, and SMAD3 mutations to 14.8% in primary sporadic CRCs.

Lin et al.\cite{54} described that nitrate 1 (NIT1) suppresses the proliferation of CRC cells through a positive feedback loop between NIT1 and the TGFβ/SMAD signaling pathway because SMAD3 transcriptionally upregulates at the transcriptional level. NIT1 belongs to the carbon-nitrogen hydrolase superfamily and plays an important role in the suppression of CRC.

Role of SMAD4

A key component of TGF-β signaling is SMAD4, which plays an important role as a so-called switch in deciding whether to stop the cell cycle or progress to the spread of cancer\cite{32}. Impaired TGF-β signaling...
due to the deletion of SMAD4 is detected in 16%-25% of CRCs[35]. Sadeghi et al[36] found SMAD4 mutations in 33.3% of analyzed tissues collected from patients with CRC.

Most SMAD4 mutations occur in the MH2 domain, although this domain represents only 41.5% of the coding sequence of the entire SMAD4 protein[36-38]. The MH2 domain is essential for homodimerization and heterooligomerization with SMAD2 or SMAD3 proteins. Therefore, mutations in this region can cause blocks to the growth, inhibition, and apoptosis that is otherwise generally induced by TGF-β. Moreover, SMAD4 mutations promote inflammation by TGF-β and thus may expand genetically damaged cells during tumorigenesis[36]. The most frequent mutation of the SMAD4 gene has been described in CRC which leads to the formation of a salt bridge between Arg361 and Asp351 and which affects homodimerization and heterooligomerization with SMAD2 and SMAD3[39,60].

Sadeghi et al[36] further described in their publication that the other significant mutations in CRC are at codons 264 and 271 of SMAD4 protein, which are located in the linker domain, a region required for subcellular localization and transcriptional activation.

Analyzes of tissue sections by immunohistochemical methods of carcinomas from various organs, including the gastrointestinal tract have shown a loss of SMAD4 expression in > 50% of colorectal carcinomas, which is associated with lymph node metastases. SMAD4 loss has been seen in 58% of pancreatic adenocarcinomas, 27% of appendiceal adenocarcinomas, 16% of cholangiocarcinomas, 10% of lung adenocarcinomas, and < 5% of esophageal, breast, gastric, and mucinous ovarian adenocarcinomas [61]. Although the LOH on chromosome 18q can be the main cause of SMAD4 loss in CRC, there are other posttranscriptional and posttranslational mechanisms that may contribute to SMAD4 protein loss or dysfunction, such as ubiquitination, sumoylation, or interference with regulatory microRNA (miRNA)[62].

Regarding the correlation between SMAD proteins and clinicopathological characteristics, Yang et al [63] showed that SMAD4 concentrations in CRC patients were significantly higher in the N0 stage compared to patients with NI stage. Regarding patients in advanced stages (TNM III-IV), reduced concentrations of SMAD4 were recorded in them compared to patients in early stages (TNM I-II). In addition, SMAD4 was significantly decreased in patients who were older than 65 years.

Szeglin et al[64] determined probes and corresponding genes from analysis of SMAD-binding elements (SBEs) that were correlated with SMAD4 expression. They subsequently confirmed that a SMAD4-modulated gene profile predicted disease-free survival in stage II and III CRC. According to them, this gene profile has prognostic potential in selected CRC patients.

**Role of SMAD7**

SMAD7 acts as an inhibitor of SMAD in the TGF-β/SMAD pathway and may prevent TGF-β-dependent SMAD2/SMAD4 complex formation and inhibit SMAD2 accumulation in the nucleus (Figure 4). SMAD7 may also promote the dephosphorylation and inactivation of TGF-βR1 with cooperation of the E3 ubiquitin ligase SMURF1/2. SMAD7 may also localize to the nucleus and limit the binding of the SMAD2-3/SMAD4 complex to specific SMAD-responsive DNA sequences[65]. So, SMAD7 plays an important role in both the cytoplasm and the nucleus, thereby maintaining the balance in the TGF-β induced signaling pathway. Inactivation of any component in this pathway will result in accelerated cell growth and dysregulation of apoptosis signals, leading to uncontrolled cell growth and differentiation, and the induction of cancer cells[66]. Therefore, overproduction of SMAD7 leads to significantly decreased EMT in response to TGF-β[67].

Several studies have reported the significant role of SMAD7 in sporadic CRC. According to results published by Li et al[66], SMAD7 is a target of miR-424, which is implicated in the regulation of SMAD7 expression via the circTBL1XR1/miR-424 axis.

Boulay and colleagues, in 264 biopsy samples from CRC patients, showed that the deletion of SMAD7 is less common than deletion of SMAD4 and SMAD2, and patients with such a SMAD7 deletion have a significantly better prognosis than patients without a deletion. Their findings demonstrated that patients with SMAD7 deletions had a low ratio of death risk and relapse, which clearly defined SMAD7 as a negative prognostic marker in CRC patients[68,69].

SMAD7 and SMAD4 genes are deregulated in CRC, whereas there is a markedly higher increase in SMAD7 expression (~ 11.3-fold) than SMAD4 expression (approximately 2-fold) in tumor cells[70]. SMAD7 protein expression is closely related to Dukes’ stage, CRC invasion depth, and lymph node metastases, and positively correlates with CRC expression[66].

Less frequently, it has been reported that SMAD7 also has an anti-cancer effect. Gastrointestinal carcinomas, such as CRC, are characterized by frequent alterations in SMAD components. Furthermore, depending on the stage of the tumor, SMAD7 activity can transition from tumor-suppressive to tumor-promoting (i.e., early vs advanced). Given the opposing roles of TGF signaling, these seemingly contradicting functions are not surprising[71,72].
of the human genome consists of non-coding RNAs (ncRNAs) that do not encode proteins. ncRNAs are divided into two larger groups\cite{73}; in one are the housekeeping ncRNAs, including the very abundant rRNAs and tRNAs, and in the other are the regulatory ncRNAs, including long ncRNAs (lncRNAs), microRNAs (miRNAs), circular RNAs (circRNAs), PIWI-interacting RNAs, small tRNA-derived RNAs (tRFs), small nuclear RNAs (snoRNAs), siRNAs and others. The most studied classes of ncRNAs are lncRNAs, miRNAs, and circRNAs. These types of ncRNAs very significantly regulate or are regulated by the TGF-β signaling pathway\cite{74}.

**LNCRNAs AS REGULATORS IN CRC**

lncRNAs influence gene expression through several mechanisms, such as silencing of the X chromosome, modification of chromatin, imprinting of the genome, activation of transcription, and nuclear transport. Imbalance in regulation of lncRNA transcription has been associated with apoptosis, angiogenesis, proliferation, invasion, metastasis and drug resistance of CRC\cite{74}.

The lncRNAs cancer susceptibility candidate 9 (CASC9) and small nucleolar RNA host gene 6 (SNHG6) can positively regulate the TGF-β pathway in CRC. CASC9, in particular, increases the stabilization of TGF-β2 mRNA\cite{75}, and a study by Zhang et al\cite{76} showed that it targets miRNA-542-3p and could also increase chemoresistance. The lncRNA SNHG6, on the other hand, targets miR-26a-5p and increases the resistance of CRC cells to 5-fluorouracil (5-FU).

The lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) has been verified to participate in the development and progression of colon cancer\cite{77}.

CTBP1-AS2 has an important role in CRC proliferation and metastasis. While CTBP1-AS2 has been shown to significantly promote activation of the TGF-β/SMAD2/3 signaling pathway, miR93-3p (a downstream molecule of CTBP1-AS2) has been shown to target the 3'-untranslated region (UTR) of TGF-β. Furthermore, investigations of the functionally of miR-93-5p showed that its overexpression exerts an anti-cancer effect by inhibiting the TGF-β/SMAD2/3 pathway\cite{78}.
miRNAs AS REGULATORS IN CRC

miRNA regulates the transcription of genes encoding proteins at the post-transcriptional level. They perform this task by binding to complementary sequences located in the 3′-UTRs of their target mRNAs [79]. miRNAs are also competitively inhibited by lncRNAs [24].

In TGF-β signaling, miRNAs can play a stimulatory role, as shown in cells treated with anti-metabolites and anti-microtubule medicines; this is similar to what has been reported in cases of chemoresistance against DNA damaging agents. In particular, miR-423-5p, miR-552, miR-34a, and the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) are examples of miRNAs that regulate TGF-β signaling in CRC. Furthermore, SMAD2, SMAD4, and TGF-βRII genes are markedly associated with miR-155 and miR-22, both of which strongly correlate with tumor properties, suggesting clinical utility in immunotherapy [24]. miR-4666-3p and miR-329 act as tumor suppressor genes, affecting TGF-βRI and thus preventing the activation of the TGF-β/Smad pathway [80]. Finally, miR-147 overexpression has been shown to inhibit EMT and the TGF-β/SMAD pathway in colon cancer cells [81].

circRNAs AS REGULATORS IN CRC

circRNAs are formed by back-splicing of linear RNA and connections via covalent linkage. circRNAs can prevent miRNAs from binding to the 3′-UTR sequence of a particular gene, by attachment to miRNAs, ultimately regulating gene expression by activating mRNA cleavage or subsequent translation [82].

circPTEN1 is significantly downregulated in CRC and its expression is positively correlated with patient prognosis. circPTEN1 binds to the MH2 domain of SMAD4 and prevents the interaction between SMAD4 and SMAD2/3, which leads to suppression of translocation of the SMAD complex into the nucleus, followed by the activation of the transcription of downstream genes that regulate the EMT by the TGF-β signaling pathway [83].

circPACRGL acts as a miR-142-3p/miR-506-3p sponge to promote TGF-β1 expression and, thus, promote the differentiation of N1 to N2 neutrophils [84].

Gaining a more comprehensive understanding of the role of ncRNAs in CRC may lead to new approaches in the treatment of this disease; however, currently, only a limited number of identified and characterized IncRNAs and circRNAs with a confirmed regulatory role in CRC are known. There remains an urgent need to investigate the role of other IncRNAs and circRNAs that may facilitate the prognosis, diagnosis and treatment of CRC.

TREATMENT OF CRC

Over the last 10 years, researchers have developed a new anticancer therapy for patients with advanced or metastatic cancer. Several recent studies have shown that drug resistance in the treatment of various cancers, including CRC, is associated with the activation of TGF-β signaling [24]. 5-FU, an anticancer agent that belongs to the category of antimetabolites, is widely used to regulate metabolic pathways that are essential for cancer cell proliferation and survival. 5-FU is a standard chemotherapeutic used for the treatment of CRC patients, and a large proportion of these patients relapse or metastasize during the course of treatment. In patients with CRC, drug resistance is a key cause of chemotherapy failure and disease progression [85,86]. Recent research suggests that SMAD4 expression levels correlate with the prognosis and response to 5-FU and can help guide therapeutic decisions regarding its administration [87,88]. Reduced concentrations of SMAD3 or loss of SMAD4 suppress the expression of tumor suppressor genes that are induced by the TGF-β signaling pathway, which in turn leads to the expression of anti-apoptotic proteins Bcl-2 and Bcl-W [89]. Increased survival of cancer cells resistance to 5-fluorouracil in CRC [89].

The role of TGF-β/SMAD signaling in tumor radiotherapy is controversial. It has been described in some studies that fibrosis is induced by upregulation of SMAD2/3 after radiation exposure. Reactive oxygen species (ROS) are involved in irradiation (IR)-induced fibrosis through TGF-β signaling. SMAD molecules that are activated by the TGF-β signaling pathway regulate ROS production by upregulating NADPH oxidase [89,90]. Mutations in some genes, such as tumor protein p53, Ras, SMAD4, and EMT, are important in radioresistance or radiosensitization and can be controlled by SMAD-dependent or SMAD-independent TGF-β pathways [91]. Publications in recent years suggest that TGF-β signaling through various mechanisms, especially through miRNA-mediated regulation, plays an important role in the resistance of tumor cells to DNA-damaging agents. In CRC, miR-34a interacts directly with the 3′-untranslated region of SMAD4 and suppresses TGF-β/SMAD4 signaling. In patients with oxaliplatin-resistant CRC, miR-34a is downregulated to increase macroautophagy by activating the TGF-β/SMAD pathway [92,93].
ANTI-TGF-β THERAPIES

The objective of targeting TGF-β signaling as a therapeutic approach to treat cancer is supported by a plethora of findings from genetic and preclinical studies. Several strategies have been tested thus far that aim to block the TGF-β signaling pathway (Figure 5). These include: (1) Preventing TGF-β production or expression of its receptor by antisense oligonucleotides (ASOs; short synthetic single-stranded nucleic acids that bind to RNA to regulate gene expression); (2) preventing TGF-β activation via integrin-blocking antibodies, in which the antibodies compete with the TGF-β ligand to bind to its receptor, as well as the ability to block the activation of latent TGF-β (both steps are crucial for TGF-β to elicit its protumorigenic and immunosuppressive responses); (3) inhibiting the interaction between TGF-β and its receptor with neutralizing antibodies to TGF-β, blocking antibodies to TGF-βRII or ligand traps (engineered soluble forms of the receptor that compete with the cell-bound receptor); (4) preventing intracellular TGF-β receptor signal transduction via small-molecule kinase inhibitors, which bind to the ATP-binding domain of TGF-β kinase and inhibit ATP kinase activity, thereby blocking the downstream signaling cascade[94]; (5) immune checkpoint inhibitors (ICIs), which have essential roles in modulating the immune system. This group includes monoclonal antibodies that send inhibitory signals to T cells, enhancing T cells’ antitumor immune response and improving antitumor defense. In addition to immunoregulatory cells such as regulatory T cells (Tregs), M2 macrophages, and myeloid-derived suppressor cells (MDSCs), the cytokine TGF-β also has the ability to control and modulate T cell functions. This is facilitated by the release of molecules that are able to activate specific ICIs. In this way, activation of inhibitory immune checkpoints, such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death-1/Ligand (PD-1/PD-L1), lymphocyte-activation gene 3 (LAG3), or T-cell immunoglobulin-and mucin domain-3-containing molecule 3 (TIM-3) can disrupt cytotoxic T-lymphocyte (CTL) proliferation in CRC and reduce the immune response against cancer[95]; (6) vaccine-based approaches to modulate TGF-β signaling, which have been applied with the aim of facilitating the immune destruction of cancer cells in many different tumor types. It is important to realize that tumors are able to prevent the activation of the immune system by hiding tumor cell antigens and also suppress the immune system. Thus, cancer vaccines will help to activate and maintain an anti-tumor immune response; and (7) adoptive cell therapy, which is a form of passive immunotherapy that involves transferring immune cells or molecules to the host[96].

Many of these agents have been or are being evaluated in clinical trials to treat CRC (Table 3).

SMALL MOLECULE INHIBITORS OF SMAD EXPRESSION AND PHOSPHORYLATION

Since SMAD molecules have an important role in the TGF-β signaling pathway, great efforts have been made for the search of SMAD activation inhibitors. Indeed, it has been shown that SMAD3 silencing can suppress cancer cell growth and metastasis by increasing the cancer-killing activity of natural killer (NK) cells. Thus, the selective inhibition of the SMAD3 protein with a potent, low toxicity drug could provide a promising anticancer treatment. Some compounds have shown good inhibitory activity against SMAD 2 or SMAD3 through direct or indirect downregulation of their respective expressions and phosphorylations[97].

Peptide aptamers or DNA aptamers are artificial short peptides, respectively single-stranded DNA or RNA nucleotides, which are antibody-like in function. Aptamers can bind specific molecules with high specificity and affinity. SMAD2-and SMAD3-binding aptamers have also been established. Upon binding to SMAD2 or SMAD3, the aptamer prevents their binding and complex formation, thereby arresting TGF-β signaling[98,99]. Aptamers also have the potential to be used more frequently in clinical practice, from disease diagnosis to targeted delivery of therapeutic agents. Their simplicity in manufacturing and lengthy shelf life significantly improve this potential[100].

The specific inhibitor of SMAD3 (SIS3) is a synthetic substance that specifically inhibits the phosphorylation of SMAD3 and thus its binding to SMAD4[101]. Furthermore, targeting the inhibition of SMAD3 is currently considered a promising therapeutic strategy in the treatment of cancer[102].

MEDICATION THERAPEUTIC STRATEGIES THROUGH THE TGF-β/SMAD SIGNALING PATHWAY

The effects of several potential molecules that induce tumor growth or inhibit the proliferation and metastasis of carcinoma cells through regulation of the TGF-β/SMAD signaling pathway have been described[103]. Baicalin is a major flavonoid, originally extracted from the edible medicinal plants of Scutellaria baicalensis and S. lateriflora. Baicalin reduces the concentrations of phosphorylated SMAD2 and SMAD3, without affecting the total levels of SMAD2 and SMAD3 and thus inhibits the TGF-β/SMAD2/3 signaling pathway in fibroblasts in vitro and in vivo without affecting SMAD 1, 5, and 8 in the BMP signaling pathway[104].
Ginseng is valued as the most important medicinal plant in traditional Chinese medicine. The major constituents of ginseng are ginsenosides. Ginsenoside Rg3 has an inhibitory effect on the TGF-β/SMAD and ERK signaling pathways in keloid fibroblasts and increases mRNA expression levels in SMAD7 [105]. Dai et al [106] showed that ginsenoside Rb2 can inhibit the expression of SMAD4 and phosphorylated SMAD2/3 in CRC cells.

Kaempferol is a natural flavanoid, a type of flavonoid, found in a variety of plants and plant-derived foods, including kale, beans, tea, spinach, and broccoli. It binds to the TβRI, leading to its inactivation. This results in inhibition of the TGF-β/SMAD signaling pathway due to reduced phosphorylation of SMAD2/3[107].

Loureirin B, a flavonoid extracted from Dracaena cochinchinensis, is used in traditional Chinese medicine (TCM). Loureirin B upregulates the expression of MMP-1, MMP-3, MMP-9, and MMP-13 and thus causes degradation of extracellular matrix, inhibits the phosphorylation of SMAD2 and SMAD3 and thus effectively suppresses the TGF-β/SMAD pathway[108].

Galangin is a polyphenolic compound derived primarily from different medicinal herbs, the effect of which is the downregulation of SMAD2 and SMAD3 phosphorylation without altering the expression of total SMAD2, SMAD3, SMAD4, SMAD6, and SMAD7[109].

Celastrol is a pharmacologically active substance extracted from Tripterygium wilfordii Hook F, which is used in TCM to treat cancer and other inflammatory diseases[110]. Zhang et al [111] showed that celastrol reduces the levels of SMAD4 and phosphorylated SMAD2/3. Together, celastrol may inhibit suppression of TGF-β signaling in CRC.
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Figure 5 Inhibition strategies of transforming growth factor-β signaling pathway and miRNAs targets for colorectal cancer treatment. TGF: Transforming growth factor; SMAD: Small mothers against decapentaplegic homolog.

CRC through TGF-β, which is a promising treatment for CRC.

Qingjie Fuzheng granules are TCM comprising a 4-herb mixture, composed of *Hedyotis diffusa* Willd, malt, *Astragalus*, and *S. barbata* D. Don significantly inhibits the expression of several key proteins in the canonical TGF-β/SMAD pathway, including TGF-β, phosphorylated SMAD2/3, and SMAD4. This inhibition leads to a decrease in the ratio of N-cadherin to E-cadherin, indicating that EMT is inhibited [111].

CONCLUSION

Antitumor immunity is mediated by macrophages, NK cells, granulocytes (polymorphonuclear leukocytes, PMNs), T cells, and antibodies. In recent years, the particular role of PMNs in regulation of adaptive immunity, especially in cancer, has emerged. PMNs in cancer are functionally diverse, with some authors describing their antitumor activity, but the number of publications in which the authors confirm their negative regulation of immune responses and their presence in cancer patients associated with poor prognosis and therapeutic outcomes is increasing. These cells suppress the physiological role of T and B lymphocytes and NK cells, and also promote tumor progression and metastasis through non-immune mechanisms. Cytokines produced by tumor cells [vascular endothelial growth factor (VEGF), TGF-β] also play a similar role when they inhibit T cell development and function. TGF-β, as an immunosuppressive factor, significantly affects the proliferation, activation, and differentiation of immune effector cells. Epigenetic changes that may be affected by the TGF-β pathway in CRC should be carefully studied because the mechanisms of drug resistance are different between patients in different stages of cancer and personalized treatment is more effective. Therefore, knowledge of the activation and inhibition of factors that affect the TGF-β signaling pathway is very important.
ACKNOWLEDGEMENTS

This review is a summary work consisting of the results of many authors. We would like to thank all the authors whose work we have included in this review article and apologize to the authors whose relevant work has not been included in this review article.

FOOTNOTES

Author contributions: Maslankova J, Vecurkovska I, Rabajdova M, Katushova J, Kicka M, Gayova M, and Katush V contributed equally to the study’s conception, design and undertaking, and to manuscript preparation; all authors have read and approve the final manuscript.

Conflict-of-interest statement: The authors declare having no conflict of interests for this article.

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S-Editor: Chen YL
L-Editor: A
P-Editor: Zhang XD

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Immunological mechanisms of fecal microbiota transplantation in recurrent *Clostridioides difficile* infection

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**Specialty type:** Gastroenterology and hepatology

**Provenance and peer review:** Invited article; Externally peer reviewed.

**Peer-review model:** Single blind

**Peer-review report’s scientific quality classification**
- Grade A (Excellent): A
- Grade B (Very good): B
- Grade C (Good): C
- Grade D (Fair): D
- Grade E (Poor): 0

**P-Reviewer:** Chen Q, China; Tieranu CG, Romania; Zhang F, China

**Received:** March 12, 2022
**Peer-review started:** March 12, 2022
**First decision:** April 6, 2022
**Revised:** May 6, 2022
**Accepted:** August 16, 2022
**Article in press:** August 16, 2022
**Published online:** September 7, 2022

**Abstract**

Fecal microbiota transplantation (FMT) is a successful method for treating recurrent *Clostridioides difficile* (C. difficile) infection (rCDI) with around 90% efficacy. Due to the relative simplicity of this approach, it is being widely used and currently, thousands of patients have been treated with FMT worldwide. Nonetheless, the mechanisms underlying its effects are just beginning to be understood. Data indicate that FMT effectiveness is due to a combination of microbiological direct mechanisms against *C. difficile*, but also through indirect mechanisms including the production of microbiota-derived metabolites as secondary bile acids and short chain fatty acids. Moreover, the modulation of the strong inflammatory response triggered by *C. difficile* after FMT seems to rely on a pivotal role of regulatory T cells, which would be responsible for the reduction of several cells and soluble inflammatory mediators, ensuing normalization of the intestinal mucosal immune system. In this minireview, we analyze recent advances in these immunological aspects associated with the efficacy of FMT.

**Key Words:** Fecal microbiota transplantation; Immunity; Mechanism; Dysbiosis; Pseudomembranous colitis; *Clostridioides difficile*
Core Tip: Fecal microbiota transplantation (FMT) is an excellent treatment option of pseudomembranous colitis due to *Clostridioides difficile* infection (CDI) because of its remarkable effectiveness. Moreover, FMT is a promising therapy for several other disorders in which dysbiosis is an important pathological factor. The mechanisms of FMT have begun to be dissected and include the restoration of the commensal microbial community structure and the modulation of several components of the immune system. This minireview focus on the FMT immune-related mechanisms for CDI.

Citation: Soveral LF, Korczaguin GG, Schmidt PS, Nunes IS, Fernandes C, Zárate-Bladés CR. Immunological mechanisms of fecal microbiota transplantation in recurrent *Clostridioides difficile* infection. *World J Gastroenterol* 2022; 28(33): 4762-4772
URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4762.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4762

INTRODUCTION

The human microbiota is a complex community of microorganisms that reside on the skin and mucosal surfaces, with gut microbiota being by far the most studied microbial subcommunity[1]. Firmicutes and Bacteroidetes are the most prevalent phyla in the human gut, followed by Actinobacteria and Proteobacteria[2]. Interestingly, mammals directly or indirectly receive signals from the microbiota for adequate development and functioning throughout life[3]. These signals are important for several systems of the human body. The interaction of the microbiota with the immune system is probably the best example of how important the commensal microbiota is for the host, given that the absence of microbiota results in an immune system with fewer and less varied components, as well as delayed immune responses[3,4]. Moreover, the presence of a normal microbiota restricts the colonization of pathogens by direct and indirect mechanisms. This function of the microbiota is known as colonization resistance[5,6]. Furthermore, the alteration of intestinal microbiota composition is called dysbiosis and commonly results in disease development.

*CLOSTRIDIOIDES DIFFICILE* INFECTION AND INTESTINAL DYSBIOSIS CORRECTION WITH FMT

*Clostridioides difficile* (*C. difficile*) is a spore-forming bacillus with the capacity to retain crystal violet staining, denoting that its cell wall is rich in peptidoglycans and, therefore, becomes positive in the staining procedure created by Hans Christian Gram in 1884[7]. Although *C. difficile* could be part of the intestinal commensal microbiota, toxin-producing strains are pathogenic. Nonetheless, the ingestion of toxin-producing *C. difficile* does not necessarily result in disease development because the microbiota is able to avoid colonization and overgrowth of this pathobiont[8]. However, *C. difficile* infection (CDI) is well known to occur due to a combination of two factors: (1) Ingestion of the bacillus spores during hospitalization, where the circulation of strains capable of expressing toxins A, B, and C - which damage the intestinal epithelium - is more common; and (2) Receiving or having recently received broad-spectrum antibiotic therapy, which will cause intestinal dysbiosis[8,9]. Thus, antibiotic exposure followed by acute episodes of diarrhea is the main clinical indicator of CDI. The detection of toxins associated with colonoscopic and/or histopathologic findings will confirm the diagnosis of pseudomembranous colitis[10]. Elderly persons are more affected by the disease; however, CDI is becoming more frequent in younger populations and with no association with previous hospitalizations[8,11]. The emergence of hypervirulent and antibiotic-resistant *C. difficile* strains contributed to the burden of worldwide cases of antibiotic-associated diarrhea and pseudomembranous colitis[8,10]. In fact, CDI may range from mild or self-limiting diarrhea to severe cases and the development of sequelae, including toxic megacolon and fulminant colitis. CDI is commonly treated by antibiotics (Metronidazole, Vancomycin, and Fidaxomicin) with efficacy rates ranging from 76% (Metronidazole) to as high as 97% (Vancomycin and Fidaxomicin)[12,13]. However, as with many other broad-spectrum antibiotics, *C. difficile* can also develop resistance mechanisms to these and other antibiotics[14]. Furthermore, antibiotic therapy, which treats CDI, will enhance dysbiosis and will predispose the patient to CDI relapse[15]. In fact, it is well known that 20%-30% of antibiotic-treated CDI cases subsequently develop recurrent episodes of the infection (rCDI)[16-18].

FMT is primarily indicated for treating pseudomembranous colitis due to rCDI[19,20]. The use of FMT for rCDI is based on several studies reporting the effectiveness of FMT, supporting it as the most effective treatment for this disease. In a systematic review on FMT effectiveness against rCDI that included 45 studies (36 cohort studies and nine randomized clinical trials), it was shown that FMT has
Fecal transplant and immunological mechanisms

91% effectiveness after eight weeks of repeated treatment - far superior to the use of antibiotics[21]. According to the United States Food and Drug Administration, FMT may be performed after two failed courses of antibiotics[22]. The fecal material for FMT may be obtained from a relative or unrelated donor and administered using a nasogastric or nasoduodenal tube or by colonoscopy[19,20,23,24]. More recently, successful FMT treatments using lyophilized solutions and capsules have been reported[25-27]. Commonly, the administration of one or two courses of FMT results in clinical remission as early as one day after the first FMT[25,24,28]. Its effects are based mainly on the restoration of eubiosis[29]. This implies that FMT effectiveness relies on microbiologic mechanisms, or in other words, the restoration of colonization-resistance-related mechanisms[30-32]. However, indirect mechanisms of colonization resistance include the crosstalk with different components of the immune system, which will be important for both maintaining the integrity of the intestinal mucosa or restoring that integrity if the disease is already present, as is the case of pseudomembranous colitis due to CDI[9,33].

Notably, FMT restores the capacity of the microbial community to convert primary bile acids (BAs) into secondary BAs, such as deoxycholic acid and ursodeoxycholic acid, which can inhibit C. difficile germination and epithelial apoptosis[34]. Although not directly shown in FMT, the optimal biotransformation of BAs by microbiota also modulates the repertoire and functions of colonic RORγt+ T regulatory (Treg) cells, contributing to intestinal homeostasis[35]. Moreover, higher levels of primary BAs in the stool, such as taurocholic acid - which can promote the spore germination of C. difficile - have been reported in rCDI patients compared to healthy individuals as well as compared to patients experiencing their first episode of CDI[36]. This is compatible with the bile salt hydrolase (BSH) gene abundance reduction - which metabolizes BAs - in rCDI patients compared to healthy and first episode CDI individuals[34]. Furthermore, BSH functional activity is rapidly restored in rCDI patients after FMT[23]. Taken together, these data indicate that gut microbial BAs metabolism is one of the molecular mechanisms of FMT to successfully treat rCDI.

Similarly, the recovery of microbiota functions after FMT is linked to the repopulation of short-chain fatty acids (SCFAs) producer bacteria - mainly members of the Clostridiales clade that include several butyrate producers[37]. SCFAs are known to serve as the main source of energy for colonocytes but also play a role in homeostasis maintenance, inducing the differentiation into effector and Treg cells in the intestinal lamina propria (LP)[38]. BAs and SCFAs are bacterial metabolites with pleiotropic effects on the immune system but, acting together, they may play a crucial role in reducing the inflammation in the intestine after FMT[35].

One important aspect of FMT refers to means of improving it by using simpler preparations that could offer more standardized formulations, being more patient-friendly, and avoiding any type of potential risks by not using an undefined combination of living microorganisms, as is the case with FMT. In this regard, Feuerstadt et al[39] have recently reported the use of oral capsules composed of live purified Firmicute bacterial spores in a phase 3 clinical trial of patients with rCDI. Of the 89 patients treated with this formulation and followed for eight weeks, they observed recurrence in 11 patients (12%) compared to 37 patients (40% of recurrence) in the placebo group[39].

On the other hand, Zhang et al[40] proposed to submit the fecal material of standard FMT to a combinatorial method of filtration and centrifugation to offer a safer, more precise and quality-controllable microbiome transplant. The authors called this material “washed microbiota transplantation” (WMT) and provided evidence of reduced levels of pro-inflammatory molecules such as leukotriene B4, corticosterone, and prostaglandin G2 in mice which were intraperitoneally injected with WMT. Despite that washed microbiota has been used successfully to treat ulcerative colitis and Crohn’s disease since 2014, it has not been evaluated in the context of rCDI[40].

Interestingly, Ott et al[41] showed that a single administration of sterile fecal filtrate (FFT), which contains bacterial components, bacteriophages, and bacteriocins but not whole bacterial cells, was able to eliminate symptoms and avoided the recurrence of CDI in 5 patients. This finding could overturn the necessity of living bacteria and successful engraftment of donor microbiota to reach the protective effect of FMT in rCDI patients. A possible explanation for this result could be that bacteria cell wall components and DNA fragments, which remain after filtration, stimulate the host’s innate immune responses, with subsequent reprogramming of the mucosal immune mechanisms against the pathogen while promoting the restoration of homeostasis. The authors also proposed an additional explanation in which the massive transfer of bacteriophages from the donor to the host would be able to correct dysbiosis in rCDI patients. Although the study does not assess BAs or SCFAs content in the FFT, it is reasonable to consider that these metabolites could also participate in the effects reached by FFT since they are expected to persist after the filtration process. The absence of potential bacterial pathogens in the transplanted material - as is the case when using FFT - could represent an important advantage for the use of FFT in immunodeficient patients instead of living bacteria. In addition, FFT could also be better standardized. Therefore, FFT needs to be explored in detail in a larger group of patients and compared to FMT. Figure 1 summarizes these FMT variations and their key features.

Taking together all these studies, one may conclude that immune pathways activated during the response to C. difficile are important not only to identify the mechanisms that effectively contribute to its elimination but also to determine which immune components are activated or respond to FMT.
Figure 1 Advantages and disadvantages of non-classical preparations methods of donor’s fecal material prior to fecal microbiota transplantation. 1Classical preparation consist in dissolve donor’s fecal material by blending with saline water and filter out residual solid feces through gauze or fabric. 2Isolation of different bacteria strains directly from donor’s fecal material. 3Basically, this method consists in consecutively centrifugation of microbiota from donors to remove the supernadants. 4Uses filtration systems to retain debris and bacterial load from the donor’s fecal material. SCFAs: Short-chain fatty acids; AMPs: Antimicrobial peptides; BAs: Bile acids.

ESSENTIAL CONCEPTS OF THE IMMUNE RESPONSE DURING CDI

As there are recent and excellent reviews on the immune response to C. difficile,[10,42,43] in this section, we present the main characteristics of this host-pathogen interaction.

The immune response to C. difficile is characterized by the development of an inflammatory reaction with Th1 and Th17 components. This response starts with bacterial sensing by epithelial cells and the release of interleukin (IL)-1 and IL-8 with high capacity to attract neutrophils.[44,45] Type-1 innate lymphoid cells (ILC-1) also participate in the response by secreting interferon-γ (IFN-γ).[46] Antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs), are important to capture and process C. difficile antigens, migrate to draining lymph nodes, and activate specific T cells.[47] Under these circumstances, Th1 cells are generated, but the secretion of IL-6 and IL-23 provide sufficient stimuli for the expansion of Th17 cells.[48] While these aspects of the immune response could be pivotal for appropriate enhancement of several bacteria-killing mechanisms by innate cells, it is already known that an exacerbated immune response signifies the development of pseudomembranous colitis, which is the histopathological lesion caused by the inflammatory response taking place in the colon.[49] To avoid the development of an immunopathological response, two important branches of immunity are required. First, the activation of Treg cells with the secretion of immune regulatory cytokines IL-10 and transforming growth factor-β (TGF-β)[50]. The source of these regulatory cytokines may also be enriched from other cell subsets such as intestinal epithelial cells, for example, which have the ability to secrete relevant quantities of TGF-β.[50,51] Secondly, recent studies have indicated the importance of active Th2 components present in patients with CDI who do not develop histopathological lesions but, instead, resolve the infection. These elements include mainly ILC-2 and eosinophils as the main cell populations[52,53], as well as type 2 cytokines, including IL-4, IL-5, IL-13, IL-25, and IL-33[54-56].

While the participation of Th1, Th17, and Treg cells during the response to bacteria with the characteristics of C. difficile is easy to understand, the type 2 component - which appears to have remarkable importance for the host to avoid an overreacting inflammatory response - is unexpected. Therefore, the antigenic components responsible for the activation of ILC-2 and eosinophils during CDI are new essential factors to be identified to better understand the effective immune response against C. difficile.
They found that innate and adaptive LPMCs stimulated with FMT-derived microbiota produced less IL-1β, tumor necrosis factor-α, and IFN-γ pro-inflammatory cytokines when compared to healthy donors. FMT reduced IL-1β, TNF-α and IFN-γ and increased IL-10 in a dose-dependent manner. Furthermore, FMT strongly reduced IL-1β, TNF-α and IFN-γ, normalized populations of ILC-2, ILC-3, F4/80+ macrophages and CD11b+Ly6G+ neutrophils. Blockade of IL-10 resulted in reduction in colon length, increased weight loss and expression of IL-1β, TNF and IFNγ genes.

The microbiota depletion due to the antibiotic treatment also resulted in a reduction of T cells with memory/effector phenotype (CD44hi), Tregs, and co-stimulatory molecules in DCs, with the restoration of all these parameters after FMT. The authors also found that IFN-γ, IL-17, IL-22, and IL-10-producing T cells decreased with antibiotic treatment but were restored with FMT. Subsequently, using the classical dextran sodium sulfate (DSS) colitis model, Burrello et al.[58] provided a more profound and dynamic analysis of the effects of FMT in resolving intestinal inflammation. They used the CXCR6egfp reporter mice, in which T cells may be tracked, including the invariant natural killer T cell population. The investigators treated mice with DSS for seven days and, after a two-day recovery period, the mice received FMT on three consecutive days. The mice received a preparation of intestinal mucus on the first day and feces from healthy donor mice on the second and third days. Evaluations were performed one and five days after the last FMT. They found that FMT reduced the production of IL-1β and increased the production of antimicrobial peptides (Camp and S100A8) and mucins (Muc1 and Muc4), all in the colonic epithelium. In parallel, they observed effects on the innate and adaptive immune systems in DSS colitic mice treated with FMT. In the innate immune system, DSS inflammation induced the expansion of ILC-2 and ILC-3, F4/80+ macrophages, and CD11b+Ly6G+ neutrophils, followed by a reduction of these populations after FMT. Furthermore, FMT strongly reduced MHC-II+ cells, indicating that the bacteriotherapy also affected APCs. These observations were correlated with the evaluation of LP mononuclear cells (LPMCs) stimulated with FMT or DSS-derived microbiota, in vitro. They found that innate and adaptive LPMCs stimulated with FMT-derived microbiota produced less IL-1β, tumor necrosis factor-α, and IFN-γ pro-inflammatory cytokines when increasing the production of IL-10. Moreover, the investigators went further, attempting to determine...
Figure 2 The immune response during recurrent *Clostridioides difficile* infection and after fecal microbiota transplantation treatment. A: During recurrent *Clostridioides difficile* (*C. difficile*) infection, the depletion of commensal microbiota results in higher levels of primary bile acids (BA$s$). These molecules are known to trigger the *C. difficile* vegetative state and its expansion, resulting in production of toxin (Tcd) A, TcdB, and TcdC, which cause apoptosis of enterocytes and release of interleukin (IL)-1 and IL-8 in the lamina propria (LP). Consequently, there is extensive recruitment of neutrophils, dendritic cells, and macrophages and type 1 innate lymphocytes (ILC-1) in an attempt to cope with the infection. These cells produce pro-inflammatory cytokines including IL-6, IL-23, interferon (IFN)-γ, which induce Th17 and Th1 differentiation. This inflammatory state dominated by IL-17 and IFN-γ promotes tissue damage, which could spread along the intestines, and is accompanied with absence of innate ILC-2 and reduction of peripheral T regulatory (Treg) cells; B: The therapeutic effects of FMT involve...
the reestablishment of a wide variety of commensal microorganisms that directly and indirectly antagonize C. difficile. Commensal strains that produce secondary Bas and short chain fatty acids are re-established, as well as the production of antimicrobial peptides by epithelial cells together with the reconstitution of the barrier integrity. These effects allow to reduce the activation of innate immunocytes, the expansion of Treg cells which produce IL-10, and subsequent normalization of Th1 and Th17 cell frequencies in the LP, IL: Interleukin; FMT: Fecal microbiota transplantation; TNF: Tumor necrosis factor; INF: Interferon; SCFAs: Short-chain fatty acids; AMPs: Antimicrobial peptides; TGF: Transforming growth factor; pBAs: Primary bile acids; sBAs: Secondary bile acids; DCs: Dendritic cell; ILC: Innate lymphoid cells; cCDI: Recurrent Clostridioides difficile infection; Treg: T regulatory; Macs: Macrophages.

the importance of IL-10 secretion for the beneficial effects of FMT to treat DSS colitis. Using a concomitant administration of IL-10 blockade in DSS mice during the FMT treatment, the authors demonstrated the pivotal importance of this regulatory cytokine for inflammation resolution, including the normalization of the histological score and intestinal weight[58].

More recently, Littmann et al[59] were able to confirm the importance of IL-10-producing cells for the host to be able to respond effectively to FMT. Their 2021 report started by considering that if the host immune system is not important for FMT to resolve the intestinal inflammation during CDI, then immune-deficient mice should be equally effective as wild-type animals to respond to FMT. To test their hypothesis, they used Rag1 double knockout animals (Rag1<sup>-/-</sup>), which lack T and B cells, and compared CDI course before and after FMT. They observed that CDI persisted in Rag1<sup>-/-</sup> animals but not in wild-type (WT) littermates nor Rag1<sup>Het</sup> controls. Importantly, they confirmed this observation by excluding the possibility that the microbiota composition of Rag1<sup>-/-</sup> mice, which is known to be different from the microbiota of Rag1<sup>Het</sup> mice, was indeed the reason for the differential responses to FMT. They were able to do so by performing three additional control experiments. First, they analyzed and compared the microbiota composition of Rag1<sup>-/-</sup>, Rag1<sup>Het</sup>, and WT mice. Second, they transferred WT or Rag1<sup>-/-</sup> microbiota to antibiotic-treated Rag1<sup>-/-</sup> and Rag1<sup>Het</sup> mice. Third, they employed germ-free C57BL/6 mice, which were cohoused with Rag1<sup>-/-</sup> or Rag1<sup>Het</sup> mice. These mice were treated with antibiotics, infected with C. difficile, and then treated with FMT. All these experiments confirmed that the difference in the responses to FMT in Rag1<sup>-/-</sup> or Rag1<sup>Het</sup> depended on an adaptive immune cell population and not on differences in microbiome composition prior to FMT. Next, the investigators focused on determining which adaptive immune cell population is important for FMT efficacy. By using specific knock-out animals, they excluded the importance of B cells, CD8<sup>+</sup> T cells, Th17, and Th1 CD4<sup>+</sup> cells. In contrast, the transient specific ablation of Treg cells using the diphertheria toxin Foxp3-DTR mice demonstrated that Treg cells are pivotal to observing the effects of FMT against CDI. Moreover, Littman et al[59] also showed that FMT engraftment is different depending on the immune activation status of the host, as well as that the microbiota post-FMT is metabolically distinct depending on the functionality of the host immune system.

Finally, Monaghan et al[60] performed a systems biology-based study to interrogate the interaction among microbiome-metabolome-immune system in the context of FMT applied to patients with severe or fulminant CDI. They studied four patients unresponsive to antibiotic therapy and treated with sequential FMT. Three patients were responders against one non-responder. The evaluations included microbiome and associated metabolome profile in fecal samples as well as the evaluation of blood samples to access the epigenomic, metabolomic, glycomic, immune proteomic, immunophenotyping, functional immune assays, and the T cell receptor repertoire. Although the small sample size did not allow the authors to draw clear conclusions, they suggest that immunosenescent signals could be associated with non-responsiveness to FMT, since they found strong correlations between peripheral senescent T cells and host factors including butyrate, serum hydroxybutyrate, fecal urso- iso- and hyodeoxycholic acids, serum immunoglobulin G Fc N-glycopeptides, and microbial taxa including Pseudomonas at the genus level[60]. The main findings of the studies recently discussed are listed in Table 1.

In summary, the studies described here indicate that FMT effectiveness rely not only on the capacity of donor eubiotic microbiota to be able to expulse C. difficile, but also to produce key metabolic products as secondary BAs and SCFAs. These metabolites, together with the displacement of the pathogen which represents less injury and consequently reduction of pathogen-derived antigens for innate and adaptive immunity activation, allow Treg cells to expand and increase the production of IL-10. This regulatory activity becomes pivotal for different types of immune cell populations and their produced cytokines to return to normal levels dampening inflammation, as shown in Figure 2.

**CONCLUSION**

The findings discussed here provide a new perspective on the therapeutic effects of FMT to restore eubiosis. This ability refers, in our opinion, two key aspects: (1) The material to be transplanted must contain the appropriate elements to displace the pathogen and modulate the immune system of the patient effectively; and (2) The status of the immune system of the patient is decisive at the moment of receiving the transplant, which means that the patient’s immune system must be able to respond
adequately to the FMT stimuli.

FMT is an effective option for the resolution of rCDI and is being used around the world increasingly. Moreover, recent studies show its efficacy in treating the first episode of CDI as well as its repeated use can treat severe and fulminant CDI forms[60]. Although the concept of the method is simple, it is a labor-intensive procedure and requires the acceptance of the patient to be treated with this kind of transplant, even if FMT-derived capsules are used. Nonetheless, as FMT is increasingly showing its benefits in a variety of clinical situations (e.g., autism spectrum disorders, type 2 diabetes, and, of course, different types of inflammatory bowel diseases), this should guarantee not only the continuing use of FMT but also the advancement of basic research seeking to identify the molecular microbiological components that are pivotal for FMT efficacy. In addition, the new evidence discussed here shows the importance of disclosing in detail the immunological pathways that must be activated/deactivated during the FMT process. At the same time, it is necessary to consider that several of these observations came from animal studies, and where FMT was used to treat colitis induced by DSS or antibiotic cocktails but not by C. difficile infection. Nonetheless, all these efforts should lead to the identification of molecular factors that may become candidates for the development of new and more conventional therapeutic products that could replace FMT in the future or improve its results.

FOOTNOTES

Author contributions: Several LF, Korczaguin GG, and Schmidt PS collected the literature and wrote the first draft, conceptualized the table and figures, and contributed equally to this work; Nunes IS and Fernandes C corrected the first draft; Zárate-Bladés CR conceptualized the structure of the text and critically revised the manuscript for important intellectual content; and all authors read and approved the final version of the manuscript.

Supported by the grant "Programa de ciência tecnologia e inovação aos grupos de pesquisa da Universidade Federal de Santa Catarina", FAPESC (2021TR000301); Several LF is a graduate student fellow of Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina, FAPESC (2003/2021); Schmidt PS is student fellow of Programa Institucional de Iniciação Científica e Tecnológica, PIBIC of the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (2021/949248); Nunes IS is a graduate student fellow of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES (202003075).

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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Fecal transplant and immunological mechanisms


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Albumin administration in patients with cirrhosis: Current role and novel perspectives

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Abstract

Mortality in cirrhosis is mostly associated with the development of clinical decompensation, characterized by ascites, hepatic encephalopathy, variceal bleeding, or jaundice. Therefore, it is important to prevent and manage such complications. Traditionally, the pathophysiology of decompensated cirrhosis was explained by the peripheral arterial vasodilation hypothesis, but it is currently understood that decompensation might also be driven by a systemic inflammatory state (the systemic inflammation hypothesis). Considering its oncotic and nononcotic properties, albumin has been thoroughly evaluated in the prevention and management of several of these decompensating events. There are formal evidence-based recommendations from international medical societies proposing that albumin be administered in individuals with cirrhosis undergoing...
large-volume paracentesis, patients with spontaneous bacterial peritonitis, those with acute kidney injury (even before the etiological diagnosis), and those with hepatorenal syndrome. Moreover, there are a few randomized controlled trials and meta-analyses suggesting a possible role for albumin infusion in patients with cirrhosis and ascites (long-term albumin administration), individuals with hepatic encephalopathy, and those with acute-on-chronic liver failure undergoing modest-volume paracentesis. Further studies are necessary to elucidate whether albumin administration also benefits patients with cirrhosis and other complications, such as individuals with extraperitoneal infections, those hospitalized with decompensated cirrhosis and hypoalbuminemia, and patients with hyponatremia.

Key Words: Cirrhosis; Albumin; Paracentesis; Spontaneous bacterial peritonitis; Acute kidney injury; Hepatorenal syndrome

INTRODUCTION

Cirrhosis and chronic liver diseases rank as the conditions with the 10th highest mortality rate worldwide[1]. Deaths are mostly related to the development of clinical decompensation of cirrhosis, and 4%–12% of individuals with cirrhosis present with at least one episode of decompensation annually[2]. This is why preventing and treating decompensating events in cirrhosis are constant concerns of gastroenterologists and hepatologists.

Albumin administration has been studied in the prophylaxis and management of different forms of decompensation of cirrhosis for many years. Albumin is exclusively synthesized by hepatocytes, and it is characterized as a water-soluble, negatively charged, 67-kDa protein, with a half-life of approximately 20 d in normal conditions. It is the most abundant protein in serum and in extracellular fluids, and it has multiple roles, including oncotic, antioxidative, detoxifying, anti-inflammatory, endothelium stabilizing, and immunomodulatory functions[3]. Traditionally, most decompensating events of cirrhosis were explained by the peripheral arterial vasodilation hypothesis[4], and albumin was considered potentially useful mainly due to its oncotic property, as it is responsible for 75% of plasma oncotic pressure[3]. However, with the current understanding that decompensation of cirrhosis is at least partly driven by a systemic inflammatory state (the systemic inflammation hypothesis)[5-8], the nononcotic properties of albumin have gained much attention[3].

This article reviews the current role and novel perspectives for albumin administration in cirrhosis. Table 1 shows the main indications for which there are formal recommendations for the use of albumin in cirrhosis as well as other potential situations in which albumin may play a role.

LARGE VOLUME PARACENTESIS

Large volume paracentesis (LVP) is the current standard of care for the management of refractory and tense ascites due to its efficacy and low rate of complications[9-11]. However, the drainage of large volumes of ascitic fluid increases cardiac output and reduces peripheral vascular resistance and effective circulating volume, leading to arterial hypotension, acute kidney injury (AKI), hepatic encephalopathy (HE), worsening of hyponatremia, and decreased survival rates. This severe condition is termed paracentesis-induced circulatory dysfunction (PICD) and is defined by a rise of more than 50% in the basal plasma renin activity a few days after the procedure, indicating the detrimental effect of volume depletion on effective volemia[12-15].
Several randomized controlled trials (RCTs) have shown that PICD can be prevented by intravenous human albumin administration, particularly in cases of paracentesis exceeding 5 L and that albumin is more effective than other plasma expanders[13,16-18]. In the seminal study by Ginés et al[13] for instance, 105 patients were randomized to be submitted to paracentesis with or without albumin infusion, and individuals receiving albumin developed less episodes of hyponatremia ($P < 0.01$) and renal impairment ($P < 0.05$).

In 2012, a meta-analysis of 17 RCTs, including 1225 patients with ascites undergoing LVP, showed that in comparison to alternative treatments albumin reduced the incidence of PICD (odds ratio = 0.39, 95% confidence interval (CI): 0.27-0.55), hyponatremia (odds ratio = 0.58, 95% CI: 0.39-0.87), and mortality (odds ratio = 0.64, 95% CI: 0.41-0.98), which appeared to be definitive evidence regarding the role of albumin infusion in LVP[19]. However, in 2019 another meta-analysis, including 25 RCTs, revisited this issue. According to this systematic review, there was no evidence of significant reduction in mortality or renal impairment when any volume expansion was compared to no volume expansion at all, but it should be highlighted that only one and two RCTs using albumin actually contributed to the analyses of these outcomes, respectively. When albumin was compared to other plasma expanders, there were significant benefits of using albumin regarding prevention of PICD [risk ratio (RR) = 1.98, 95% CI: 1.31–2.99] and hyponatremia (RR = 1.49, 95% CI: 1.03–2.14), but there was no evidence of significant differences between treatments regarding renal impairment (RR = 1.17, 95% CI: 0.71–1.91) and mortality (RR = 1.03, 95% CI: 0.82–1.30)[20].

The use of vasoconstrictors, such as vasopressin, midodrine, and noradrenaline, has been proposed as an alternative to albumin in order to overcome the marked arterial vasodilation and arterial hypotension associated with PICD, but evidence on the clinical utility of such drugs is limited in this context. An RCT compared the effect of midodrine and standard albumin doses in preventing PICD in 50 patients with cirrhosis and tense refractory ascites. Midodrine therapy was associated with higher incidence of AKI, worsening of hyponatremia, and higher plasma renin activity and plasma aldosterone concentration, suggesting that this drug is not as effective as intravenous albumin in preventing PICD after LVP[21].

Despite the existence of some doubts concerning the benefits of albumin on hard outcomes (renal impairment and mortality), its clear benefits on important surrogate outcomes (PICD and hyponatremia) allow albumin to be recommended in patients with cirrhosis undergoing paracentesis of more than 5 L. According to the European Association for the Study of the Liver, it should be administered at a dose of 8 g/L ascitic fluid removed[9], while the American Association for the Study of Liver Diseases recommends it is used at doses of 6–8 g/L ascites removed[11].

Modest volume paracentesis (< 5 L) seems to have less serious impacts on hemodynamic and neurohumoral systems, and therefore it might be safe to perform the paracentesis without administering albumin[22]. The exception to this seems to apply to patients with acute-on-chronic liver failure (ACLF) undergoing paracentesis < 5 L because these individuals usually have an intense hemodynamic impairment that theoretically increases the risk of PICD. A recent study randomized 80 subjects with ACLF undergoing paracentesis < 5 L to receive standard doses of albumin or no fluid expansion and demonstrated that PICD was significantly more common in the control group than in the albumin group (70.0% vs 30.0%, $P = 0.001$), with significantly higher incidences of HE (50.0% vs 27.0%, $P = 0.04$), hyponatremia (67.5% vs 22.5%, $P < 0.001$), AKI (62.5% vs 30%, $P = 0.001$), and short-term mortality (62.5% vs 27.5%, $P = 0.003$)[23].

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**Table 1 Current recommendations and potential indications for albumin administration in cirrhosis**

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1Recommendations for albumin administration according to the European Association for the Study of the Liver[9] and the American Association for the Study of Liver Diseases[11].
AKI AND HEPATORENAL SYNDROME

AKI is a common complication of cirrhosis, reported in up to one-third of hospitalized patients with advanced liver disease[24-27]. The diagnostic criteria of AKI in cirrhosis have evolved over the years and are currently based on an acute increase in serum creatinine by ≥ 0.3 mg/dL or ≥ 50% from baseline [28]. The new definition and classification proposed by the International Club of Ascites has allowed for earlier recognition of AKI and implementation of therapeutic strategies, such as intravenous albumin use.

Hypovolemia accounts for about one-half of all cases of AKI in cirrhosis, and it is often driven by excessive use of diuretics and/or fluid losses from lactulose-induced diarrhea. In addition to diuretic withdrawal, intravenous albumin at 1 g/kg/d (maximum 100 g/d) for 2 d has been recommended for volume expansion, especially in patients with AKI stage ≥ 1b[9,11] since mortality appears to significantly increase from this stage on[24,29-32]. Response failure to a 2-d fluid challenge with albumin is suggestive of hepatorenal syndrome (HRS), formerly classified as type 1 and currently as HRS–AKI, once structural kidney injury has been excluded. Recent history of shock, nephrotoxic drugs, proteinuria, microhematuria, and hydronephrosis on renal ultrasound must be ruled out for the diagnosis of HRS–AKI, which is one of exclusion[9,11,28,33].

The benefits of albumin in HRS, a functional kidney injury driven by reduction in renal blood flow, extend beyond just plasma volume expansion. This has been demonstrated in a study comparing albumin with hydroxyethyl starch, a synthetic colloid, in patients with spontaneous bacterial peritonitis (SBP). Administration of albumin resulted in significant improvement on systemic hemodynamics, whereas this effect was not appreciated in the starch group[34]. Furthermore, albumin carries important anti-inflammatory and antioxidant properties, and it has been shown to bind circulating bacterial products, thus preventing their negative consequences on the systemic circulation[35,36]. This has led to the extension of albumin use past the initial fluid challenge at a recommended dose of 20–40 g/d[9,11,29]. Although albumin alone has a limited role in HRS–AKI[37,38], the benefit of added albumin to vasoconstrictor therapy in HRS has been demonstrated in one nonrandomized study comparing terlipressin with terlipressin plus albumin. Despite being a small study, it demonstrated that the combination therapy group had a significantly higher response rate compared to terlipressin monotherapy (77% vs 25%, P = 0.03)[39].

It is important to note that excessive albumin use in AKI and HRS can be detrimental and contribute to development of pulmonary edema and respiratory failure. This concern has been raised in the recently published CONFIRM study, a large RCT comparing terlipressin with placebo[38]. Concomitant albumin was given in > 80% of patients in both arms at a mean total dose of 200–240 g over 5 d (40–50 g/d). A higher incidence of respiratory failure was observed in the terlipressin group (14% vs 5%), presumably secondary to pulmonary edema because of the known cardiovascular effects of terlipressin in combination with excessive albumin use[40]. Thus, volume status should be closely monitored in these patients and judicious albumin use is recommended.

SBP

Bacterial infections occur in 25%–35% of the patients hospitalized with advanced cirrhosis[41], and they are associated with increased morbidity and mortality[42,43], particularly when acquired in the hospital or in healthcare facilities, due to the presence of multidrug resistant organisms[44-48]. SBP is frequently reported as the most common infection in subjects with cirrhosis and ascites[44,45] and one of the main precipitants of ACLF[49]. It is diagnosed in the presence of a polymorphonuclear cell count > 250/mm³ in the ascitic fluid, and it is usually treated with third-generation cephalosporins in those patients with community-acquired infection[9,11].

However, since the publication of the pivotal RCT by Sort et al[50] in 1999, it is clear that this infection should not be treated exclusively with antibiotics. In that study, 126 hospitalized patients with SBP were randomized to receive intravenous cefotaxime versus intravenous cefotaxime plus albumin administered at a dose of 1.5 g/kg body weight at baseline, followed by 1 g/kg on day 3. The authors described a threefold reduction in the incidence of renal impairment favoring the albumin group (33% vs 10%, P = 0.002). More importantly, 3-mo mortality was also significantly lower in the albumin group (41% vs 22%, P = 0.03), which was attributed to the decrease in the incidence of AKI[50].

The benefit of using albumin in SBP was initially attributed to plasma expansion and/or prevention of circulatory dysfunction[34,50,51], but it actually seems that albumin infusion leads to a reduction of plasma levels of nitric oxide, tumor necrosis factor-α, endotoxin and interleukin-6[52]. These findings favor the concept that albumin is more than a colloid and that it has anti-inflammatory properties in individuals with decompensated cirrhosis[53].

In the study by Sort et al[50], renal impairment was negligible in both groups of patients in the presence of baseline bilirubin levels < 4 mg/dL and serum creatinine level < 1 mg/dL, suggesting that albumin administration might be restricted to higher-risk subjects. Nevertheless, subsequent data have disputed these findings[54,55]. In this regard, a meta-analysis including four RCTs[34,50-52] evaluated...
the role of albumin in SBP and concluded that albumin was associated with a lower incidence of renal impairment and mortality, not identifying a significant difference in albumin effects according to baseline levels of bilirubin or renal function[56]. Since then, several international guidelines recommend the use of high-dose albumin in patients with SBP, even in patients at lower risk for renal impairment[9, 11,57]. However, there still is some controversy regarding albumin dosing and schedule since the use of lower doses of albumin were associated with a reduction in proinflammatory cytokines in an RCT[52], and no major differences in outcomes were observed in a subsequent Brazilian trial comparing standard versus lower doses of albumin in SBP[58].

EXTRAPERITONEAL INFECTIONS

Infections (not only SBP) characterize state 6 (end-state) in the clinical course of cirrhosis[59], as they increase the risk for AKI, HRS, organ failure, and ACLF[60]. It has also been recently demonstrated that infections are the most important precipitating factor for acute decompensation of cirrhosis, even in patients without ACLF[61]. On the other hand, in compensated cirrhosis, the role of infections is not completely understood. A recent cohort study has demonstrated that 17% of patients with compensated cirrhosis developed infections, particularly respiratory and urinary tract infections, which led to decompensation of cirrhosis in 26% of cases and to an increased mortality rate[62].

Therefore, considering the importance of infections in the prognosis of cirrhosis, improving the efficacy of therapeutic strategies would be of the utmost importance. In SBP, as previously discussed, the addition of albumin to antibiotic treatment represented an important improvement in the therapeutic strategy[50], and it was natural to study if a similar intervention could reach the same results in extraperitoneal infections. The rationale behind this proposal is that albumin is a multifunctional protein, which also has important nononcotic properties, as previously mentioned[3]. Furthermore, individuals with cirrhosis are not only quantitatively deficient in albumin but also qualitatively, which highlights the concept of the effective albumin concentration[63,64].

Some RCTs have evaluated the subject of albumin administration in infections other than SBP. In the first of them, patients with cirrhosis and extraperitoneal infections were randomized to receive antibiotics plus albumin (same doses as for SBP) or antibiotics alone, and albumin led to improved circulatory and renal functions. In that study, there was no significant difference in 3-mo survival between groups, but albumin use was an independent predictive factor of survival after adjusting for other factors[65].

In the second RCT, despite delaying the onset of renal failure, albumin was not able to significantly reduce its incidence or improve survival. Besides, 8.3% of subjects receiving albumin developed pulmonary edema as a complication[66]. It is noteworthy, however, that the study had important methodological limitations.

In the third RCT, albumin was associated with a higher resolution of ACLF as well as with lower incidence of nosocomial infections. Nevertheless, once again, there was no significant difference between groups regarding mortality[67]. An important limitation of this study is the fact that only 23% of the estimated sample was actually enrolled in the trial[68].

In order to further examine this subject, our group has performed a meta-analysis of RCTs evaluating the role of albumin in extraperitoneal infections. In that meta-analysis, there was no evidence of significant benefit of albumin in reducing renal dysfunction (RR = 0.55, 95% CI: 0.25–1.19, \( P = 0.13 \)) or mortality in 30 d (RR = 1.62, 95% CI: 0.92–2.84, \( P = 0.09 \)) and 90 d (RR = 1.27, 95% CI: 0.89–1.83, \( P = 0.19 \))[69]. Therefore, at this moment, a general recommendation cannot be made regarding the administration of albumin in patients with cirrhosis and extraperitoneal infections[9]. Still, there might be a role for albumin in a subgroup of extraperitoneal infections, particularly the most severe of them[70].

LONG-TERM ALBUMIN ADMINISTRATION

Ascites is the most common among severe complications of cirrhosis[71], and it marks state 4 in the natural history of this disease[59]. Ascites is associated with a 5-year mortality of 50%, and persistent ascites predicts mortality independently of the Model for End-Stage Liver Disease score[59,72]. Therefore, strategies aiming at the increase in survival of patients with cirrhosis and ascites are constantly pursued.

Long-term albumin administration has been studied in the management of patients with cirrhosis and ascites for many decades. The rationale for its use relies on the hypothesis that albumin could reduce effective arterial hypovolemia through plasma expansion and that the nononcotic properties of albumin could act against the systemic inflammation that is behind decompensation of cirrhosis[3,5]. Once again, the concept of effective albumin concentration should be highlighted in this context[63]. As albumin is quantitatively and qualitatively deficient in cirrhosis, leading to alterations in the transport and metabolism of substances as well as to the impairment of systems associated with the redox balance, inflammation, and coagulation, it is hypothesized that albumin supplementation could prevent...
the decompensation of cirrhosis[73-77].

Five RCTs evaluated the role of long-term albumin administration in patients with cirrhosis and ascites. The first study evaluated a small sample of subjects with persistent ascites already under treatment with diuretics. Albumin was used at doses of 25–100 g every 1–2 d according to serum colloid osmotic pressure, and 25–100 g every 1–2 wk thereafter. The osmotic pressure was improved in individuals receiving albumin, but mortality was not different between groups[78].

After that, two Italian RCTs evaluated the matter. Gentilini et al[79] enrolled patients with ascites unresponsive to a low-sodium diet, while Romanelli et al[80] studied individuals with their first episode of grade 2–3 ascites. Both studies randomized patients to receive albumin at doses of 25 g every week for 12 mo and every other week thereafter[79,80]. In the former study, albumin led to significantly lower cumulative probabilities of recurrence of ascites and hospitalization, but there was no benefit regarding mortality[79]. In the latter, though, patients receiving albumin had a significantly lower probability of recurrence of ascites and a higher cumulative survival rate[80].

Finally, in 2018, two more RCTs on long-term albumin administration were published. Solà et al[81] evaluated albumin at doses of 40 g twice a month in combination with midodrine for patients with cirrhosis and ascites in the waiting list for liver transplantation, but there was no significant difference in survival or in complications of cirrhosis between study groups. The high rate of transplantation in that study might have led patients to be treated with albumin for an insufficient period of time (since they were quickly transplanted). The fact that the renin-angiotensin-aldosterone system activity did not completely normalize in subjects receiving albumin also supports the hypothesis that higher doses and longer duration of albumin administration might have been necessary[81].

On the other hand, Caraceni et al[82] randomized patients with persistent ascites to receive albumin 40 g twice a week for 2 wk and once a week thereafter or no plasma expansion. Individuals receiving albumin had significantly better results than their counterparts regarding mortality, need for paracentesis, SBP, extraperitoneal infections, HE, renal dysfunction, HRS, hyponatremia, and hyperkalemia[82].

Considering the differences in the results of these studies, we have performed a meta-analysis on this issue. Pooling the data from all five RCTs, it was demonstrated that albumin significantly reduced recurrence of ascites/need for paracentesis (RR = 0.56, 95%CI: 0.48-0.67, P < 0.001). There was also a trend towards a lower risk of mortality favoring albumin, but it did not reach statistical significance (RR = 0.88, 95%CI: 0.67-1.14, P = 0.33). There was no evidence of significant differences between groups regarding refractory ascites, SBP, HE, gastrointestinal bleeding, or adverse events[83].

We understand the main reason for the study by Caraceni et al[82] having reached such outstanding results relates to the doses of albumin used. In a recent study, the effects of different doses of long-term albumin administration were compared. While high-dose albumin (1.5 g/kg/wk) led to normalization of serum levels of albumin, improvement of circulatory and cardiac function, and reduction in plasma levels of cytokines, low-dose albumin (1.0 g/kg every 2 wk) did not[84]. It is noteworthy that the trial by Caraceni et al[82] was the one using the highest dose of albumin among the five RCTs on this issue, and, even so, the dose used was only slightly higher than that considered insufficient in the abovementioned study[84]. Moreover, while changes in serum levels of albumin were not different between groups in the trial by Solà et al[81], the intervention group had a normalization of serum albumin in the study by Caraceni et al[82], which reinforces the idea of insufficient doses of albumin in the former trial.

Furthermore, in a post hoc analysis of the study by Caraceni et al[82], the authors demonstrated that a serum level of albumin of 4 g/dL at 1 mo should be the target in long-term albumin administration in order for the highest survival rates to be achieved. In that publication, the authors suggested the hypothesis of the albumin gap, associating the amount of albumin required not only to baseline albumin levels but also to the severity of liver disease and highlighting the importance of the concept of effective albumin concentration[63].

Therefore, considering the abovementioned evidence, we believe that long-term albumin administration in patients with cirrhosis and ascites will probably become a formal recommendation in the near future. It must be emphasized, though, that the most recent guidelines still did not include this indication of albumin use in cirrhosis[11,85]. Hopefully, the ongoing PRECIOSA trial (NCT03451292) will provide us with more definitive data on this issue.

OTHER INDICATIONS

Decompensated cirrhosis

A recent RCT (the ATTIRE study), including 777 patients hospitalized for decompensated cirrhosis with baseline serum albumin levels < 3 g/dL, evaluated the effects of increasing these levels by daily intravenous administration of albumin. In that study, albumin administration was not superior to placebo in preventing a composite endpoint of infection, AKI and death[86].

Nevertheless, there are important points to consider. (1) No information on the distribution of patients between groups according to the Child–Pugh classification was provided. This is of great importance due to the recognized heterogeneity of such a group of patients. In fact, this could influence
the response to albumin infusion; (2) Ascites relates to the severity of circulatory dysfunction in patients with advanced cirrhosis. Although ascites was present in 62% of patients in the albumin group and in 71% in the control group, there was no information about its grade, the percentage of individuals with refractory ascites, use of diuretics, and their doses. Furthermore, there were no data on serum sodium concentration, another important marker of circulatory dysfunction; (3) There were no data on the severity or type of infections and their effects on circulatory function. Previous research suggests that giving albumin to patients with cirrhosis may be especially effective in the subset of patients with circulatory and renal dysfunction[50]; and (4) Finally, evidence shows that high doses of albumin are required for individuals with cirrhosis to benefit[84] and that the target level of serum albumin should be 4 g/dL[63]. Patients in the trial by China et al[86] reached levels barely over 3 g/dL. Therefore, it is likely that they did not receive enough albumin to benefit from the intervention.

The results of the ATTIRE study should be interpreted with caution, as it seems that rather than trying to make a general recommendation on albumin administration in decompensated cirrhosis, it might be more appropriate to define the best albumin administration strategy and the subgroup of patients with cirrhosis who could benefit most from its effects.

**HE**

The understanding of the pathophysiology of HE has increased in recent years. Thus, besides the traditional concept that implies cerebral exposure to ammonia as the basic mechanism for HE occurrence, new proposals suggest that the activation of inflammatory mediators, cerebral blood flow alterations due to circulatory dysfunction, and oxidative stress altogether contribute to the astrocytic injury leading to HE[87]. Albumin, a multifunctional protein, as previously mentioned in this review, could therefore play an important role in the treatment of HE.

In 2004, a pilot study evaluated the effects of plasma expansion with albumin in patients with cirrhosis and diuretic-induced HE. Albumin was more effective than a gelatin-based colloid solution in improving HE grade and lowering plasma concentrations of malondialdehyde, an oxidative stress marker[88]. After that pilot study, a multicenter, double-blind RCT found no significant differences between albumin or saline solution in the resolution of HE in patients with cirrhosis hospitalized with this complication. However, the same study demonstrated a significant improvement in 90-d survival in the albumin group (69.2% vs 40.0%, P = 0.02)[69]. In yet another RCT that compared lactulose plus albumin (group 1) versus lactulose alone (group 2) for the treatment of HE, 75% of patients in group 1 and only 53% of those in group 2 had complete reversal of HE (P = 0.03). Moreover, mortality was significantly lower in group 1 (18.3% vs 31.6%, P = 0.04)[88]. In this regard, a recent meta-analysis of RCTs aimed at clarifying the role of albumin in HE. In that study, albumin administration was able to significantly improve HE (RR = 0.60, 95%CI: 0.58–0.95, P = 0.03) and mortality (RR = 0.54, 95%CI: 0.33–0.90, P = 0.02)[91].

In another clinical context, that of the prophylaxis of TIPS-induced HE, Riggio et al[92] compared patients receiving albumin to a historical control group and found no significant difference in the incidence of overt HE[92]. However, the important methodological limitations of that study must be kept in mind when appraising its results.

Considering what was presented, despite the absence of a formal recommendation for the administration of albumin in the treatment of HE, it seems that there is initial evidence favoring its use. Further studies should be encouraged in this regard.

**Hyponatremia**

Hyponatremia is an important marker of prognosis in cirrhosis as it can induce neurological complications, and it is associated with reduced survival. Hyponatremia in cirrhosis results from a reduction in free water excretion due to nonosmotic secretion of antidiuretic hormone caused by splanchnic vasodilation. The use of albumin may decrease antidiuretic hormone secretion by improving relative hypovolemia, and therefore it might be useful in the management of hyponatremia[9].

In a small series, McCormick et al[93] observed complete reversal of hyponatremia after albumin infusion to three patients with decompensated cirrhosis. Moreover, an RCT evaluated the role of albumin infusion in hyponatremic subjects with cirrhosis, showing a significant improvement in serum sodium concentration, an increase in free water clearance, and a reduction in vasopressin concentration in the albumin group when compared to the placebo group[94]. Furthermore, in a large cohort of patients with cirrhosis and hyponatremia, albumin use was independently associated with the normalization of serum sodium levels[95]. More recently, in an analysis of the ATTIRE trial database, albumin infusions also led to an improvement in hyponatremia, which did not translate into benefits regarding hard outcomes[86]. Therefore, due to the scarcity of data, other studies are needed before albumin infusion can be recommended as a therapeutic option in patients with cirrhosis and hyponatremia.

**Cirrhotic cardiomyopathy**

Albumin seems to prevent cardiac output reduction and plasma renin activity increase in patients with cirrhosis more effectively than other plasma expanders[3]. The increase in cardiac output induced by albumin seems to be independent of volume expansion[96], which might be explained by the reversal of
the negative effects of tumor necrosis factor-alpha and oxidative stress on cardiac contractility[97].

ACLF

Considering the exacerbated systemic inflammatory state associated with the pathophysiology of ACLF and the anti-inflammatory properties of albumin, it could be hypothesized that albumin might play a role in the treatment of ACLF. There are limited data on this issue at the moment[98], but an RCT on albumin in extraperitoneal infections has demonstrated that individuals receiving this intervention had higher rates of ACLF resolution than their counterparts. Moreover, subjects receiving albumin had evidence of suppression of the systemic inflammation (decrease in white blood cells, C reactive protein, and interleukin-6), which was not specific for those with ACLF[67].

On the other hand, there remains a concern that administered albumin could be modified and added up to the pool of pathological albumin of such severely ill patients[98]. It is noteworthy that patients with cirrhosis, particularly those with advanced disease, have an impairment of albumin function in all of its domains and, therefore, a reduction in effective albumin concentration[64,76]. In this regard, research on ways of improving the quality of commercially available albumin is of the utmost importance since it could lead to an increase in albumin effectiveness as well as to a reduction in costs [3].

PREDICTION OF RESPONSE TO ALBUMIN ADMINISTRATION

Not all patients receiving albumin will benefit from it, and biomarkers capable of identifying those most likely to benefit from it would be extremely useful[3]. Effective albumin concentration, which reflects the portion of the albumin pool with normal structure and function, is superior to total albumin in stratifying individuals with compensated cirrhosis, acute decompensation, or ACLF as well as in distinguishing patients with or without complications of cirrhosis. Therefore, effective albumin concentration seems to be promising as a predictor of prognosis and treatment response in these patients. However, further studies are required not only regarding effective albumin concentration but also for other biomarkers[64,99].

ADVERSE EVENTS

Albumin infusion is generally safe, but careful evaluation of the patient is necessary in order to avoid complications, particularly volume overload and pulmonary edema[38,86]. Other uncommon complications of albumin might relate to contamination by blood-derived pathogens as well as to its administration to individuals allergic to albumin and to those with severe anemia, severe coagulopathy with pulmonary hemorrhage, or with subcutaneous bleeding[98,100].

ECONOMIC ASPECTS

Albumin administration has traditionally been considered costly. However, its cost has decreased over time. More importantly, albumin administration was proven cost-effective in different settings when evaluated using a decision-tree economic model. In that study, when compared to saline, gelatin, and no fluid expansion, albumin was the dominant treatment (more effective and less costly) for patients undergoing LVP. Regarding individuals with SBP, combining albumin and antibiotics was more cost-effective than using antibiotics alone in all three evaluated countries, and it was the dominant strategy in two of them. Finally, in HRS, combining albumin and a vasoconstrictor was the dominant strategy when compared to using a vasoconstrictor alone. Therefore, the concept of albumin administration being costly should be revisited since it is not only cost-effective but also cost-saving in most settings [101].

CONCLUSION

Due to the pathophysiological mechanisms behind decompensations of cirrhosis, albumin plays an important role in their prevention and management through its oncotic and nononcotic properties. International medical societies have made formal evidence-based recommendations for albumin administration in LVP, AKI, HRS and SBP. Promising evidence suggests that long-term albumin in patients with ascites, albumin in modest-volume paracentesis in individuals with ACLF, and albumin in HE are also probably beneficial. Further studies are needed to elucidate the role of albumin in other clinical scenarios, such as extraperitoneal infections, decompensated cirrhosis with hypoalbuminemia, and hyponatremia.
FOOTNOTES

Author contributions: All authors contributed to this paper with conception of the manuscript, literature review and analysis, drafting and critical revision of the manuscript, and approval of the final version of the paper.

Conflict-of-interest statement: All authors report no relevant conflicts of interest for this article.

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S-Editor: Ma YJ
L-Editor: Kerr C
P-Editor: Ma YJ

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Basic Study

Novel therapeutic diiminoquinone exhibits anticancer effects on human colorectal cancer cells in two-dimensional and three-dimensional in vitro models

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**Abstract**

**BACKGROUND**

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality. Cancer stem cells (CSCs) in CRC, which are spared by many chemotherapeutics, have tumorigenic capacity and are believed to be the reason behind cancer relapse. So far, there have been no effective drugs to target colon CSCs. Diiminoquinone (DIQ) has shown promising effects on targeting colon cancer. However, there is limited research on the effects of DIQ on eradicating CSCs in CRC.

**AIM**

To investigate the anticancer potential of DIQ on colon CSCs in two-dimensional...
(2D) and three-dimensional (3D) models using colonospheres and patient-derived organoids.

**METHODS**

Various 2D methods have been used to assess the effect and the mechanism of DIQ on HCT116 and HT29 cell lines including cell proliferation and viability assays, migration and invasion assays, immunofluorescence staining, and flow cytometry. The potency of DIQ was also assessed in 3D culture using the sphere formation assay and colon cancer patient-derived organoid model.

**RESULTS**

Our results showed that DIQ significantly inhibited cell proliferation, migration, and invasion in HCT116 and HT29 cell lines. DIQ treatment induced apoptosis along with an accumulation of both CRC cell lines, as well as an increase in reactive oxygen species in the sub-G1 region and in reactive oxygen species in both CRC cell lines. DIQ reduced sphere-forming and self-renewal ability of colon cancer HCT116 and HT29 stem/progenitor cells at sub-toxic doses of 1 μmol/L. Mechanistically, DIQ targets CSCs by downregulating the main components of stem cell-related -catenin, AKT, and ERK oncogenic signaling pathways. Potently, DIQ displayed a highly significant decrease in both the count and the size of the organoids derived from colon cancer patients as compared to control and 5-fluorouracil conditions.

**CONCLUSION**

This study is the first documentation of the molecular mechanism of the novel anticancer therapeutic DIQ *via* targeting CSC, a promising compound that needs further investigation.

**Key Words:** Diiminoquinone; Anticancer activity; Colorectal cancer; Cancer stem cells; Patient-derived organoids; Colonospheres

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**Core Tip:** Diiminoquinone has anticancer activity against colorectal cancer. Diiminoquinone targets chemoresistant cancer stem cells in three-dimensional in vitro models by downregulating the main components of stem cell-related -catenin, AKT, and ERK oncogenic signaling pathways. Our findings highlight diiminoquinone’s novel therapeutic potential against colorectal cancer.


**URL:** [https://www.wjgnet.com/1007-9327/full/v28/i33/4787.htm](https://www.wjgnet.com/1007-9327/full/v28/i33/4787.htm)


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**INTRODUCTION**

Colorectal cancer (CRC) ranks as the third most common cancer worldwide in 2020 in terms of incidence in men and women, and the second most common cause of cancer deaths in 2020 reaching 935000 deaths according to GLOBOCAN 2020 data[1].

Current medical treatment of CRC includes a wide array of systemic therapies, which include chemotherapeutics [such as 5-fluorouracil (5FU)], targeted therapy (such as epidermal growth factor receptor inhibitors), in addition to immunotherapy, depending on the stages of CRC. High mortality and recurrence rates of CRC are mainly correlated to metastasis, treatment resistance[2,3], and presence of chemoresistant cancer stem cells (CSCs)[4,5]. Although 5FU is the standard chemotherapy for CRC, either alone or in combination with other treatments, it has been ineffective and found to cause drug resistance[6,7]. Around 75% of patients with metastatic CRC receiving chemotherapy develop recurrence within 18 mo[8]. Although the field has witnessed several advances on the quest to control advanced and metastatic colon cancer with some newly developed drugs, there is still a pressing need to fully understand colon cancer biology, develop novel treatment approaches and pre-clinical models, and identify useful therapeutics targeting CSCs and chemoresistant cells and aiming at increasing patient survival.

Recently, we have witnessed the development of different types of in vitro three-dimensional (3D) culture systems to recapitulate the in vivo cancer growth[9,10]. 3D culture systems mainly include organoid models and multicellular spheroid models[11]. Cancer treatment, particularly using 3D culture...
for targeting CSC, is rapidly progressing toward personalized medical treatment taking into consideration the individual molecular biology and genetic variability of tumors. Introducing in vitro patient-derived organoid culture systems to 3D models have revolutionized CRC research.[12,13].

CSCs are characterized by their self-renewal, pluripotency, and tumor expansion potential of differentiated cell populations with altered molecular and cellular phenotypes.[14]. They are responsible for angiogenic induction and apoptotic resistance. This small subpopulation is associated with tumor invasion and metastasis, therapeutic resistance, cancer relapse, and poor prognosis in patients.[15]. CSCs are present within solid tumors, and they are recognized to be resistant to chemotherapies such as 5FU or oxaliplatin[4]. Intriguingly, there are no effective drugs to target CSCs in CRC. Therefore, targeting this population holds hope for treatment response.

Studies have reported that some quinones, which are often secondary metabolites derived from plants, possess anticancer activity.[16]. They are present and clinically used in a variety of cancer treatments, such as the anthracyclines daunorubicin, doxorubicin, and mitoxantrone, acting through the redox quinone-hydroquinone system.[17]. Anthraquinones are a class of natural compounds that possess anticancer properties against various skin cancer cells and breast cancer cells[18]. Studies have shown that thymoquinone, which has a basic quinone structure, induces apoptosis and halts metastasis in CRC.[19,20]. Also, iminoquinone exerts anticancer effects through inhibition of cell survival/proliferation and inhibition of oncogene expression[21].

The novel diiminoquinone (DIQ) compound has recently shown potent anticancer effects against the HCT116 CRC cell line as reported in our previous study.[22]. The activity of DIQ is believed to be based on the structural similarities between quinones and diiminoquinones. Here, we investigated the anticancer activities and targeting mechanism(s) of DIQ against human colon CSCs using colonosphere cultures and patient-derived organoids. This study represents the first comprehensive documentation of the activity of DIQ against colon CSCs, findings that will provide the basis for proposing this stable and non-toxic compound for clinical testing and future discovery of new effective treatments for patients with colon cancer.

MATERIALS AND METHODS

Cell culture condition
Human CRC cell lines HCT116 and HT29 and non-tumorigenic fetal human intestinal FH74Int cell line were purchased from ATCC (ATCC, Manassas, VA, United States). HCT116 and HT29 cell lines were cultured and maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, United States) and L-glutamine (Sigma-Aldrich). FH74Int cells were grown in DMEM (Lonza, Verviers, Belgium) supplemented with 10 μg/mL insulin and 1% sodium pyruvate. Cell culture media was supplemented with antibiotics [1% penicillin-streptomycin (100 U/mL)], 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), and 5 μg/mL Plasmocin™ Prophylactic (InvivoGen, San Diego, CA, United States). Cells were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air and were routinely checked for mycoplasma contamination. All cells were mycoplasma free.

DIQ preparation and treatment
The purified compound DIQ (Figure 1) was synthesized by Professor Makhlouf Haddadin (Department of Chemistry, American University of Beirut)[22]. Stocks of the purified compound DIQ were prepared by dissolving 5 mg in 1 mL 100% dimethyl sulfoxide (Pan Biotech, Aidenbach, Germany). DIQ dilutions were stored at -20 °C. The stock solutions were then dissolved in cell culture medium such that the percentage of dimethyl sulfoxide on cells was less than 0.1%.

MTT cell viability assay
The anti-proliferative effects of DIQ were measured in vitro by using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay according to the manufacturer’s instructions. HCT116 WT, HT29, and FH74Int cells were seeded in 96-well culture plates at a density of 10000 cells per well and incubated overnight. Then, the subconfluent cells were treated in triplicate with different concentrations of DIQ diluted in 100 μL complete media for 24, 48, and 72 h. For each time point, 10 μL of 5 mg/mL [in 1 × phosphate-buffered saline (PBS) MTT reagent was added to each well and incubated at 37 °C for 4 h. The reduced MTT dye was solubilized with absolute isopropanol (Sigma-Aldrich) (100 μL/well) after which MTT optical density was measured at 595 nm by an ELISA reader (Multiskan Ex; ThermoFisher Scientific, Waltham, MA, United States). The percent cell proliferation with respect to control was determined for each drug dose.

Trypan blue exclusion assay
HCT116, HT29, and FH74Int cell lines were seeded in duplicates in 24-well culture plates at a density of 50000, 80000, and 100000 cells/300 μL complete media per well, respectively. Cells were incubated overnight then treated in duplicate with various concentrations of DIQ (1, 4, and 10 μmol/L) for 24, 48,
and 72 h. Attached live cells were harvested by trypsin EDTA and added to the supernatant. The cell pellet was resuspended in 300 μL media. Live cells were counted using a hemocytometer. The percentage cell viability was expressed as percentage growth relative to the control condition of each time point and are derived from the mean of triplicates wells. Each experiment was repeated at least three times.

**Wound healing assay**

For wound healing, or scratch assay, CRC cells were seeded in 24-well plates and incubated until they reached 80%-90% confluence. Cells were then treated with 10 μg/mL mitomycin C (Sigma-Aldrich) to block cellular proliferation. A sterile 200 μL tip was used to scratch wounds of the same width on each monolayer. After washing the plates twice with PBS (Sigma-Aldrich) to remove the detached cells, the remaining cells were cultured in complete media with or without DIQ treatment at the IC\textsubscript{50} concentration. Images using bright-field microscopy were subsequently taken at 0 h and 72 h to compare the wound width. The wound width was measured and expressed as a percentage of the relative wound width. The experiment was repeated three times with duplicate measurements in each experiment.

**Transwell invasion assay**

For the transwell invasion assay, 0.3 × 10\textsuperscript{5} HCT116 and 0.5 × 10\textsuperscript{5} HT29 cells were seeded in serum-free medium in the top chamber of 24-well inserts (pore size, 8 μm; Falcon, ThermoFisher Scientific) coated with 1:10 dilution in cold PBS of Matrigel\textsuperscript{TM} (BD Bioscience, Franklin Lakes, NJ, United States). A medium supplemented with 10% FBS was used as a chemoattractant in the lower chamber. Cells with or without DIQ treatment were allowed to migrate through the membrane coated with Matrigel\textsuperscript{TM} at 37 °C in a 5% CO\textsubscript{2} incubator for 72 h. Non-migratory cells in the upper chamber were then gently scraped off with a cotton-tip applicator. Invading cells on the lower surface of the membrane were fixed and stained with hematoxylin and eosin. After staining, the total number of invading cells was counted using an inverted light microscope (10 × objective) from six consecutive fields for each well.

**Reactive oxygen species**

The level of intracellular reactive oxygen species (ROS) in HCT116 and HT29 was measured using the fluorescent probe dihydroethidium (DHE). For DHE staining, cells were seeded at a density of 50000 cells on coverslips in 24-well cell culture plates and allowed to become 40%-50% confluent. Following 48 h incubation with DIQ treatment at the IC\textsubscript{50} dose, CRC cells were fixed in 4% formaldehyde for 20 min. After fixation, CRC cells were washed twice with 1 × PBS, then incubated with 20 μmol/L DHE dye (Invitrogen, Carlsbad, CA, United States). After 45 min staining, the DHE stain was removed, and the cells were washed with 1 × PBS. Mounting media with 4',6-diamidino-2-phenylindole dye was added. Fluorescence images were taken immediately under a Zeiss LSM710 Laser confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with Zen software to process the images.

**Cell cycle analysis**

Cells were seeded at 5 × 10\textsuperscript{5} cells in 6-well cell culture plates and incubated overnight prior to drug treatment for 24 and 72 h. Cells were then harvested and washed in PBS then fixed in 70% ice-cold ethanol added dropwise to the cell pellet while vortexing for 30 min on ice. To ensure that only DNA was stained, fixed cells were incubated for 30 min at 37 °C with 100 μL of propidium iodide (PI) (Sigma) solution [6 μL RNase, 30 μL PI (1 mg/mL)] in the dark in a flow tube (BD Falcon, ThermoFisher Scientific). A total of 10000 gated events were acquired in order to assess the proportions of cells in different stages of the cell cycle. Cell cycle analysis was performed by flow cytometry using Guava EasyCyte8 Flow Cytometer-Millipore. GuavaSoft™ 2.7 Software.
Annexin V-PI staining
HCT116 and HT29 cells were seeded at a density of $5 \times 10^6$ cells in 6-well cell culture plates and incubated overnight prior to drug treatment for 72 h. Cells were then harvested and washed in cold PBS. The pellet was resuspended in 100 μL binding buffer and stained with 5 μL annexin V-FITC and 5 μL PI in the dark for 30 min at room temperature. Then, 400 μL binding buffer was added, and apoptotic cells were analyzed with fluorescence-activated cell sorting flow cytometry.

Sphere formation assay
Self-renewal capacity is deemed to be one of the major defining hallmarks of stem/progenitor cells. Thus, to determine whether DIQ was able to target the self-renewing CSC pool, we investigated sphere formation capability over 5 generations. The sphere formation assay was used as previously reported by our laboratory [23,24]. Briefly, 1000 single cells/well in 96-well culture plate were suspended in cold Matrigel™/serum-free medium (1:1) in a total volume of 10 μL. Cells were seeded uniformly in a circular manner around the bottom rim of the well and allowed to solidify in the incubator at 37 °C for 1 h. Subsequently, 100 μL of RPMI with 5% FBS treated with DIQ was added gently in the middle of each well. Each experimental condition was performed in duplicate. Spheres were replenished with warm media as in the original seeding every other day. Spheres were counted in the 96-well plate dishes after 8 to 12 d of sphere culture, and bright field images of the spheres were obtained using Axiovert microscope from Zeiss at × 10 magnification. Images were analyzed by Carl Zeiss Zen 2012 image software to determine sizes. Sphere-formation unit (SFU) was calculated for each generation as follows: SFU = number of spheres formed/number of cells originally plated. Results were represented as percentage of the SFU of each condition.

Immunofluorescence imaging of colonospheres
Spheres at generation 1 were collected with cold RPMI media and centrifuged to washout all Matrigel™ debris. After centrifugation, spheres were fixed in situ in 4% paraformaldehyde (PFA) at room temperature for 20 min. The PFA was aspirated gently, and spheres were permeabilized with 0.5% Triton X-100 for 30 min at room temperature. After carefully aspirating the permeabilization solution, spheres were blocked using the sphere blocking buffer [0.1% bovine serum albumin (BSA), 0.2% Triton X-100, 0.05% Tween-20, and 10% normal goat serum in PBS] for 2 h at room temperature. Spheres were washed in PBS then incubated overnight with different primary antibodies for assessment of treatment and characterization including Ki67, CD44, Gamma H2A histone family member X (γH2AX), cytokeratin (CK)19 and CK8 (refer to Table 1 for details on antibodies used). After gentle washing with PBS containing 0.1% Tween-20, spheres were incubated with Alexa-488 and/or 568-conjugated IgG (Invitrogen) for 2 h at room temperature. Spheres were mounted with the antifade Fluorogel II with 4',6-diamidino-2-phenylindole (Abcam, Cambridge, United Kingdom). Confocal fluorescent images were acquired and analyzed using the Carl Zeiss LSM 710 Laser scanning microscope.

Western blot analysis
For two-dimensional (2D) western blot results, cells were plated in 12-well plates, treated with DIQ, and then collected. For 3D western blot results, HCT116 and HT29 cells were plated in 24-well plates ($5 \times 10^5$ cells/well) with or without treatment to form spheres. At day 8-10, spheres were collected with cold RPMI media then washed with PBS to remove any residual media. Proteins were then extracted with RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology, Dallas, TX, United States). Protein extracts were quantified using the DC Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, United States) according to the manufacturer’s protocol. Equal amounts of protein lysate were mixed with 5% β-mercaptoethanol and 2X Laemmli Sample Buffer (Bio-Rad Laboratories), electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories) for 2 h. Membranes were blocked for 1 h with 5% skim milk in tris-buffered saline with 0.1% tween 20, then blotted with primary antibodies (antibodies used are listed in Table 2) overnight at 4 °C. The next day, membranes were washed three times with tris-buffered saline with 0.1% tween 20 and blotted with corresponding secondary antibodies for 1 h at room temperature. Hybridization with GAPDH-HRP (6C5) coupled antibody was performed for 1 h at room temperature as the housekeeping gene. Membranes were developed, and target proteins were detected using the enhanced chemiluminescence system (Bio-Rad Laboratories). Images were generated and quantified using ChemiDoc™ Imaging Systems (Bio-Rad Laboratories).
Table 1 List of primary and secondary antibodies used in immunofluorescent staining

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Table 2 List of primary and secondary antibodies used in western blot experiments

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**Ethical consideration of patient derived-organoid culture**

The study with all its experimental protocols was conducted under the Institutional Review Board approvals of the American University of Beirut and American University of Beirut Medical Center to obtain patient information and human colorectal tissue samples from consenting patients. All protocols were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and in agreement with all ethical considerations of the Institutional Review Board for experiments involving human subjects. Oral consent was obtained from all patients, and confidentiality was maintained. For colectomy specimens, a core biopsy was taken from the area most likely to be involved with cancer according to the surgeon and pathologist recommendations.

**Tissue processing and patient-derived organoid culture**

Colon tumor tissue from patients was rinsed with PBS and manually minced using sterile scalpels. The majority of minced fragments was employed for organoid culturing; remaining fragments were transferred directly to 4% PFA for histological examination. According to the protocol described by Boehnke et al.[25], minced fragments for organoid culturing were digested in advanced adDMEM/F12 (Gibco, ThermoFisher Scientific) supplemented with 1 × P/S, collagenase IV (Sigma-Aldrich), and amphotericin B (Sigma-Aldrich) at 37 °C for 1 h. During incubation, the tissue fragments were repeatedly suspended with a 100 μL micropipette. To exclude undigested tissue fragments, the suspension was filtered through a 100 μm cell strainer (Corning, Corning, NY, United States). The
flowthrought was subjected to consecutive filtrations when needed. Isolated cells were seeded in 24-well plates with Matrigel at a cell density of 20000 single cells/well. Then, 20 μL drops were plated into the middle of the well. The plate was placed upside down in the 37 °C incubator for 15 min to allow the Matrigel to solidify. Finally, 300 μL of prewarmed human colon growth medium plus Y-27632 was added into each well. Cells were cultured with adDMEM/F12 additional with various factors added to maintain tumor biological traits and growth activity. Medium was changed every 2-3 d. Cultures were passaged when the aggregates reached 800 μm diameter. Organoids were counted at day 8-12 of each passage under Axiocvert inverted microscope at × 10 magnification, and then images of organoids were taken at the same magnification. Images were then analyzed by Carl Zeiss Zen 2012 image software to determine size. The organoid formation count (OFC) was calculated at each generation by counting the number of organoids formed, starting with the same number of input cells in all conditions.

**Passaging of the established patient-derived organoids**
Organoids were collected when they reached the appropriate size and confluency for passaging (8-12 d after plating). To dissolve Matrigel, ice-cold medium was used, and organoids were collected. Organoids were then centrifuged at 200 g for 5 min at 4 °C. After that, the pellet was resuspended in 1 mL ice-cold adDMEM/F12 to dissolve residual Matrigel. After counting the cells in the pellet, the cells were resuspended in 90% cold Matrigel and seeded as a 5 μL drop in 96-well plate. Cells were cultured with adDMEM/F12 additional with various factors added to maintain the tumor’s biological traits and growth activity. Medium was changed every 2-3 d with or without DIQ treatment. Cultures were passaged when the aggregates reached 800 μm diameter. The previous steps were repeated for several generations.

**Cell line-derived organoid protocol**
Briefly, 5000 HCT116 and HT29 single cells/well in 96-well culture plate were suspended in cold Matrigel™/serum-free medium (9:1) in a total volume of 5 μL as drops in the middle of individual wells of 96-well culture plates. Plated colon cells were allowed to solidify in the incubator at 37 °C for 30 min. Subsequently, 200 μL/well of advanced DMEM/F12 media with several factors, with or without DIQ treatment, was added. Each experimental condition was performed in duplicate. Organoids were replenished with warm media as in the original seeding every other day. Organoids were counted in the 96-well plate dishes after 8 to 12 d of organoid culture, and bright field images of the organoids were obtained using Axiocvert microscope from Zeiss at × 10 magnification.

**Immunofluorescence and morphological analysis of colonospheres and colorectal organoids**
Spheres/organoids were collected with cold RPMI media and centrifuged to wash all Matrigel™ debris. After centrifugation, spheres/organoids were fixed in situ at 4% PFA at room temperature for 20 min. The PFA was aspirated gently, and spheres/organoids were permeabilized with 0.5% Triton X-100 for 30 min at room temperature. After carefully aspirating the permeabilization solution, spheres/or ganoids were blocked using the blocking buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, and 10% normal goat serum in PBS) for 2 h at room temperature. Spheres/organoids were washed in PBS then incubated overnight with different primary antibodies for assessment of treatment and characterization (refer to Table 1 for details on antibodies used). After gentle washing with PBS containing 0.1% Tween-20, spheres/organoids were incubated with Alexa-488 and/or 568-conjugated IgG (Invitrogen) for 2 h at room temperature. Spheres/organoids were mounted with the antifade Fluorogel II with 4',6-diamidino-2-phenylindole (Abcam). Confocal fluorescent images were acquired and analyzed using the Carl Zeiss LSM 710 Laser scanning microscope. Paraffin embedding, microtome sectioning, and standard hematoxylin and eosin staining were all performed by the Histology Laboratory at the Diana Tamari Sabbagh building; all steps were performed at room temperature.

**Animal experiments**
Animal experiments were performed according to approved protocols by the Institutional Animal Care and Use Committee of the American University of Beirut. Mice were housed under optimum conditions of temperature and light set in specific pathogen-free animal housing. Mice were kept in plastic cages covered with sawdust and had unrestricted access to a commercial mouse diet (24% protein, 4.5% fat, 4% fiber) and water. Animals were sacrificed by cervical dislocation following deep anesthesia with isoflurane. For tumor induction in mice, a group of 6-8-wk old non-obese diabetic severe combined immunodeficiency male mice were inoculated subcutaneously into the flanks with 100 HCT116-derived spheres in a total volume of 50 μL growth media and Matrigel™ (1:1). Upon the detection of a palpable tumor post cell/sphere injection, group 1 injected with 3D spheres was treated with saline (control group), and group 2 injected with 3D spheres was treated with DIQ (20 mg/kg). Mice were treated three times/wk until tumor burden necessitated that we sacrificed the animals. Tumor size was measured every other day using Mitutoyo caliper throughout the study. Mice were monitored daily for signs of morbidity. Body weight recordings were carried out biweekly.
**Statistical analysis**

All statistical tests were performed using GraphPad Prism 7 (version 7.0; GraphPad Software Inc., La Jolla, CA, United States). Student’s t test, One-way or two-way analysis of variance tests were used in this study. In all statistical tests, the mean of treated groups was compared to the mean of control groups. Statistical significance was reported at P values of < 0.05; *P < 0.05; **P < 0.01; ***P < 0.001. Experimental values were means ± standard error of the mean.

**RESULTS**

**DIQ reduced the cell proliferation of human CRC cell lines in 2D in vitro models**

To assess the effect of DIQ compound on the proliferation of human CRC cell lines cultured in 2D monolayers, we employed the MTT assay. Two human CRC cell lines, HCT116 and HT29, were treated with different concentrations of DIQ (1, 4, and 10 μmol/L) for 24, 48, and 72 h. The MTT results revealed that DIQ significantly inhibited the proliferation of HCT116 and HT29 human CRC cells at micromolar concentrations in a time- and dose-dependent manner (Figure 2A). Interestingly, a concentration of DIQ as low as 4 μmol/L was able to inhibit cell proliferation by approximately more than 30% at 24 h in HCT116 and HT29 cell lines and more than 50% cell reduction was observed at 48 h and 72 h in both cell lines. The mean IC\(_{50}\) values of DIQ was approximately 4 μmol/L in both cell lines (Figure 2A). The effect of DIQ on the viability of the human CRC cell lines was further confirmed by trypan blue exclusion method, and there was consistency between the MTT results and trypan blue exclusion assay (Figure 2B). Interestingly, DIQ treatment had relatively limited toxicity to the human non-tumorigenic intestinal FHS74Int cells when applied at doses up to 5 μmol/L and over a 72-h period (Supplementary Figure 1A).

**DIQ inhibited migration and invasion of CRC cells**

One of the most well-known properties of cancer cells is their ability to break away from their site and invade neighboring tissues[26]. Wound healing and transwell invasion assays were employed to evaluate the effects of DIQ on human CRC cell migration and invasion. DIQ at the corresponding IC\(_{50}\) concentration significantly suppressed and slowed down the cell migration ability of both cell lines at 72 h compared to the vehicle-treated control cells as determined by the wound healing assay (Figure 3A). The treatments failed to close the wound by more than 70% in both cell lines compared with control conditions, which were able to almost completely close the wound (Figure 3A). In addition, DIQ showed significant inhibitory potential on CRC cell invasion. The number of HCT116 and HT29 invasive cells were remarkably decreased in response to FBS in treated conditions reaching a value of less than two-fold compared to the control condition at 72 h (Figure 3B).

**DIQ induced cell cycle arrest and apoptosis in CRC cells**

To evaluate the underlying mechanism of growth inhibition by DIQ in CRC, the cell cycle distribution analysis of HCT116 and HT29 cells treated with the IC\(_{50}\) concentration of DIQ for 72 h was performed using flow cytometry. As shown in Figure 4A, DIQ treatment in HCT116 cells caused G1 arrest with concomitant decreases in the S and G2/M fractions mainly after 72 h. No changes in the cell cycle were noticed after treating both cell lines with DIQ for 24 h. DIQ effect on the HCT116 cell cycle was pronounced at 72 h. The proportion of HCT116 cells in G1 phase was increased from 45.6% in control cells to 60.2% in cells treated with DIQ for 72 h, while the proportion of cells in G2/M phase decreased from 35.2% to 21.5% (Figure 4A). However, in HT29 cells, DIQ treatment induced S phase (38.35%) cell cycle arrest after 72 h treatment and depleted cells at G1 and G2/M phases. Interestingly, upon treatment with 4 μmol/L DIQ, the percentage of HCT116 and HT29 cells in the sub-G1 phase significantly increased reaching 3.5- and 5.0-fold at 72 h, respectively, suggesting that the reduction in cell viability in response to DIQ could be due to cell death (Figure 4A). To further confirm whether growth inhibition was related to apoptosis, Annexin V and PI staining was performed. As shown in Figure 4A, after treating CRC cells with DIQ at the indicated concentrations for 72 h, the total apoptotic cell populations were significantly increased in both cell lines reaching 61 % in HCT116 and 70% in HT29 cells.

**DIQ induced the production of ROS in CRC cells**

Recently, targeting cancer via ROS-based mechanisms has been reported as a radical therapeutic approach[27]. To investigate the effect of DIQ on cellular stress and the involvement of oxidative stress in their anti-proliferative effect in CRC, ROS production was examined by DHE stain intensity. DHE is a fluorescent dye that can easily permeate cell membranes and has been widely used to quantify cellular O\(_2\)− and H\(_2\)O\(_2\) by producing red fluorescent products. Our results showed that a significant increase of the DHE staining intensity was observed in treated cells at 48 h as compared to the control (Figure 4B). Thus, DIQ treatment induced ROS production in both CRC cell lines.
Diiminoquinone reduced the proliferation and the viability of HCT116 and HT29 colorectal cancer cell lines in a time- and dose-dependent manner.

A: The anticancer effect of different concentrations of diiminoquinone (DIQ) on the proliferation of HCT116 and HT29 cells using the MTT assay was determined in triplicates at 24, 48, and 72 h. Results were expressed as the percentage of proliferation of the treated group compared to the control at every time point; B: The anticancer effect of different concentrations of DIQ on the viability of HCT116 and HT29 cells using the trypan blue exclusion assay was determined in triplicates at 24, 48, and 72 h. Results were expressed as percentage of viable cells of the treated group compared to the control at every time point.

Data represent an average of three independent experiments and is reported as mean ± standard error of the mean (aP < 0.05, bP < 0.01, cP < 0.001).

Diiminoquinone altered the expression of the cell cycle and proliferation markers in CRC cells

To determine the association between the observed cell cycle arrest and the increased ROS in HCT116 and HT29, western immunoblot analyses were performed on total cell extracts prepared from 2D-treated cells to detect possible changes in the expression of cell cycle and proliferation markers. As shown in Figure 4C, the expression levels of p53 and p21, which are cell cycle regulators of the G1 phase, were upregulated by 1.28-fold and 1.42-fold, respectively, in HCT116 upon DIQ treatment as compared to control conditions. Whereas, in HT29 treated cells, p53 was downregulated and p21 was significantly upregulated by 1.8-fold, suggesting that the inhibitory mechanism of DIQ is different in HCT116 and HT29 cells. The expression of the proliferation-associated proteins, such as AKT, p-AKT, ERK, p-ERK, and proliferating cell nuclear antigen (PCNA), were markedly decreased by DIQ treatment in both cell lines (Figure 4F). In addition, the expression levels of stem cell markers, CD133 and β-catenin, were also downregulated in both CRC cell lines.

Diiminoquinone targeted the enriched population of human CRC stem cells in 3D

We investigated colonosphere formation of HCT116 and HT29 cells, a salient feature of CSCs. To better visualize their sphere forming capabilities in 3D cultures, HCT116 and HT29 cells were cultured as single cells in Matrigel™ for 8-12 d in the presence of DIQ. The spheres were then visualized under an inverted light microscope, and bright-field images were taken (Figure 5). Cells that were able to form spheres in the first generation were collected and propagated by dissociating spheres into single cells and reseeding the same number of cells (1000 cells/well). The assay was performed until the fifth generation. Our data showed that both HCT116 and HT29 cells formed spheres, suggesting the presence of a unique population with stem cell-like properties. Notably, a clear dose-dependent attenuation of the SFU at generation 1 for both cell lines was observed when treated with different concentrations of DIQ (0.5 and 1 μmol/L). The SFU was always significantly and remarkably lower in drug-treated cells compared to that of the control condition by more than 50% (Figure 5). Consecutive propagations of...
Figure 3 Diiminoquinone reduced the migration and the invasion of HCT116 and HT29 colorectal cancer cells. A: HCT116 and HT29 cells were seeded in 24-well plates. A scratch was made on confluent cells using a 200 μL tip, and images were taken at 0 h and 72 h with or without the indicated treatment. Quantification of the distance of the wound closure was assessed over time. Representative images of wound healing assay at × 5 magnification (scale = 100 μm); B: Colorectal cancer cells were seeded onto the Matrigel-coated membrane in the top chamber of the transwell and were either treated or not with the indicated concentration in the presence of fetal bovine serum in the lower chamber. Cells that invaded to the lower chamber after 72 h were fixed, stained with hematoxylin and eosin, counted, and are represented as the number of invading cells compared to the control. Data represent an average of three independent experiments and is reported as mean ± standard error of the mean (*P < 0.05, **P < 0.01, ***P < 0.001). DIQ: Diiminoquinone.
control (13.3%) at generation 5 reaching approximately 1%. Moreover, as shown in Figure 5, HT29 cells were more sensitive to DIQ, and there was an eradication of spheres at generation 4 (SFU = 0%) compared to the control (14.28%). In addition to assessing its effect on self-renewal capacity, DIQ significantly decreased the sizes of the spheres by more than 50% as compared to untreated control conditions. Further decrease in sphere sizes was recognized over the 5 generations in both cell lines depicting pronounced additive effect of the treatments on the formed spheres upon propagation (Figure 5). Thus, DIQ has led to fewer and smaller HCT116 and HT29 spheres. Interestingly, DIQ treatment did not show any significant effect on the size and SFU of FHS74Int-derived spheres over 5 generations (Supplementary Figure 2B). Taken together, these findings suggest that DIQ specifically targeted the colorectal CSC.

**DIQ induced apoptosis and inhibited proliferation of human CRC stem cells**

Spheres collected at generation 1 were subjected to immunofluorescence analysis of the expression of the proliferation marker Ki67, cytokeratin epithelial markers, CK8 and CK19, and the stem cell marker CD44. Our data revealed that Ki67, CK8, and CK19 expression were significantly reduced in treated spheres derived from HCT116 and HT29 cell lines (Figure 6A-D). The downregulation of the CK19 marker in both HCT116 and HT29 spheres at generation 1 could be an indicator of an inhibition of the epithelial-mesenchymal transition process. Immunofluorescence staining showed higher expression of CD44 in control spheres at generation 1 indicating enriched stemness in these cells. Treatment with DIQ showed a significant reduction of CD44 expression in HCT116 and HT29 colonospheres as compared to the control, which is in tune with the downregulation of the CRC stem marker CD133 data (Figure 6C and E). Finally, DIQ effect on DNA damage was studied by assessing the expression of γH2AX. Our results revealed that the expression of γH2AX was markedly increased in treated spheres in both cell types (Figure 6D).

To further assess the effect of DIQ on the enriched CSCs population, we were interested in determining the effect of DIQ on the expression of proliferation markers, stem cell markers, and Wnt signaling molecules of CSCs using western blot. Consistent with the western blot analyses of 2D CRC cells, the expression of the proliferation markers p-AKT and p-ERK were remarkably downregulated by DIQ treatment in both HCT116 and HT29-derived spheres confirming inhibitory effects of DIQ on the proliferation of 3D CSC colonospheres (Figure 6E). Western blot analysis revealed a decrease in the levels of the proliferation marker PCNA post treatment consistent with the data that DIQ decreased the size of HCT116 and HT29-derived spheres (Figure 6E). For the Wnt signaling studies, we investigated treatment effects on β-catenin, which plays an important role in colon cancer stemness properties. Western blot analysis showed a downregulation of β-catenin expression in treated compared to untreated spheres. Analysis of p53 and p21 protein expression in HCT116 spheres upon DIQ treatment showed upregulation of these proteins by 1.32-fold and 1.99-fold, respectively, further confirming apoptosis induction (Figure 6E). Only p21 expression was upregulated in HT29 cells by 1.26-fold as compared to non-treated spheres, whereas the expression of p53 was not affected by DIQ treatment in...
Diiminoquinone reduced the sphere-forming and self-renewal ability of colon cancer stem/progenitor cells. A: HCT116 and B: HT29 cells were seeded at a density of 1000 single cells/well in Matrigel™ for 8 d with and without 1 μmol/L diiminoquinone (DIQ) at generation 1. Spheres were propagated for five generations in duplicates for each condition. Media or treatment was replenished every 2 d. Spheres were counted at day 8-12 of sphere culture. Results are expressed as sphere-formation unit, which was calculated according to the following formula: sphere-formation unit = (number of spheres counted/number of input cells) × 100. Quantification of the average size of generation 1 to generation 5 colon cancer spheres with or without treatment conditions. Spheres sizes were measured by Carl Zeiss Zen 2012 image software. Data represent an average diameter (μm) of 50 measured spheres. Representative bright field images of HCT116 and HT29 colon spheres in Matrigel™ taken by the Axiovert inverted microscope are shown. Data represent an average of three independent experiments and is reported as mean ± standard error of the mean (aP < 0.05, bP < 0.01, cP < 0.001).

HT29 spheres.

**DIQ had antitumor potential in non-obese diabetic severe combined immunodeficiency mice injected with HCT116 spheres.**

To investigate the antitumor effect of DIQ on targeting the CSC population of cells in vivo, we subcutaneously injected two groups of non-obese diabetic severe combined immunodeficiency mice with 100 spheres derived from HCT116 cells. Mice developed tumors in 2 wk and were then treated...
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DOI: 10.3748/wjg.v28.i33.4787 Copyright ©The Author(s) 2022.

Figure 6: Diminoquinone induced apoptosis and inhibited proliferation in colon cancer stem/progenitor cells. Representative immunofluorescence imaging of control and diiminoquinone (DIQ)-treated HCT116 and HT29 spheres collected at generation 1. A-D: Spheres stained for cytokeratin (CK8, green) and CK19 (red) (A), KI67 (B), CD44 (C), and gamma H2A histone family member X (γH2AX) (D) were obtained using confocal microscopy. The nuclei were stained with anti-fade reagent Fluorogel II with 4',6-diamidino-2-phenylindole (DAPI). The quantification of the intensity of CK8, CK19, CD44, and Ki67 stain in control and DIQ-treated spheres was performed using Carl Zeiss Zen 2012 image software. Stain intensity was normalized to size. Scale bar 20 μm; E: Analysis of p53, p21, CD133, β-catenin, proliferating cell nuclear antigen (PCNA), p-ERK, ERK, p-AKT, and AKT protein expression in HCT116 and HT29 generation 1 spheres upon treatment is shown. GAPDH served as an internal control. Bands were detected by enhanced chemiluminescence using ChemiDoc MP Imaging System. Fold expression changes normalized to GAPDH and to total ERK and total AKT in the cases of p-ERK and p-AKT expression, respectively, are given. Data represent an average of three independent experiments and is reported as mean ± standard error of the mean (aP < 0.05, bP < 0.01, cP < 0.001).

with 20 mg/kg DIQ three times per week for 21 d. DIQ treatment did not cause any change in the body weight or death of mice, indicating no toxicity.

DIQ significantly inhibited tumor growth in the treated group when compared to the control group particularly at day 21 (Figure 7). Interestingly, the average tumor volume was 403.2 mm³ in DIQ-treated mice at sacrifice, while it was 2158.5 mm³ in the control group (Figure 7).

Assessment of the effect of DIQ treatment on the established colon cancer patient derived organoids: Organoids as models for DIQ assessment

We established a 3D organoid system from fresh tissue samples obtained from different stages of 5 random colon cancer consenting patients. As described in the methods section, a total of 20000 single cells derived from freshly digested tissues were plated per 20 μL droplets of 90% Matrigel™ in 24-well plates. Cells were plated depending on the total cell count that was successfully derived from the tissue specimens. Despite the expected challenges in modeling colon cancer, we succeeded in establishing colon organoids from patients undergoing colectomy. Organoids formed at generation 1 were dissociated, propagated to generation 2, and the effect of DIQ on the organoids formed was assessed. The growth of organoids was determined by the total number (OFC) and size (diameter) of the organoids formed. The response of colon cancer patient-derived organoids to DIQ was compared to that of 5FU, which is the standard first-line treatment option for CRC. This response was evaluated on 5 random treatment-naïve patients with different clinical data. We succeeded in establishing colon organoids and propagating them. The two different doses of DIQ (0.5 and 1 μmol/L) displayed a highly significant inhibition in the OFC and the size of tumor organoids derived from the 5 studied patients when they were compared to the control group in a dose-dependent manner (Figures 8-10). These results were consistent with the response of HCT116 and HT29 cell line-derived organoids to DIQ treatment. DIQ elicited a statistically significant decrease in both OFC and size of cell line-derived organoids (Supplementary Figure 2).

In Patient 1, organoids were successfully propagated up to generation 6 as shown in Figure 8. Interestingly, an increase in the number of tumor organoids formed was observed with each propagation, thus indicating enrichment of stem cells and enhancement of the establishment of colon organoids. Characterization of the established patient 1-derived organoids was performed by studying the expression of the CRC epithelial lineage markers CK19 and CK8 and the stem cell marker CD44. Using immunofluorescence staining, the tumor organoids showed a positive expression of CK19, CK8, and CD44 confirming the presence of stem-like/progenitor CRC cells within the bulk of our patient-derived organoids.

As shown in Figure 9, a reduction in the OFC and size of the treated organoids was noticed in patient 3 with rectal mucinous adenocarcinoma (pT2 stage). Characterization of patient 3derived organoids and corresponding tissue was assessed by investigating the expression of CK19 and CD44 markers using immunofluorescence staining. These organoids mimicked the heterogeneity of corresponding tumor
Figure 7 

Diiminoquinone reduced tumor growth in non-obese diabetic severe combined immunodeficiency mice. Non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice (5 mice/group) were injected with 100 HCT116 generation 1 spheres. Tumor growth was monitored by measuring the tumor volume during 21 d of treatment (3 times per week) with either 20 mg/kg diiminoquinone (DIQ) or physiologic saline. Representative images of control and DIQ-treated mice at day 21. Data represent an average of two independent experiments and is reported as mean ± standard error of the mean ($^{a} P < 0.05, ^{b} P < 0.01, ^{c} P < 0.001$).

Figure 9 

The co-expression of CK19 and CD44 was decreased upon treatment in a dose-dependent manner (Figure 9B). In Patients 2 and 5, both exhibited similar grade (grade 2) moderately differentiated sigmoid colon adenocarcinoma and were of the same stage (pT3). DIQ treatment at concentrations as low as 0.5 μmol/L displayed a decrease in the growth of the organoids in Patient 2 and an eradication of organoids in Patient 5 (Figure 10). Organoid formation was eradicated upon DIQ (0.5 and 1 μmol/L) treatment in Patient 4, who was diagnosed with moderately differentiated (grade 2) pT2 sigmoid colon adenocarcinoma. Interestingly, the effect of DIQ on the OFC and the size of the organoids was more potent than that of 5FU particularly in patients 2, 4, and 5 (Figure 10).

DISCUSSION

In this study, we investigated the anticancer activity of DIQ in 2D and 3D models of human colon cancer. DIQ reduced the sphere forming and self-renewal ability of CRC HCT116 and HT29 stem cells at sub-toxic doses. Mechanistically, DIQ targeted CSCs by reducing the proliferation marker Ki67 and CRC stem cell markers CD44 and CK19 as well as inducing DNA damage through increasing γH2AX expression and downregulating the main components of stem cell-related -catenin, AKT, and ERK oncogenic signaling pathways. DIQ displayed a highly significant decrease in both the count and the size of the organoids derived from colon cancer patients as compared to control and 5FU conditions. Furthermore, in 2D culture, DIQ significantly inhibited cell proliferation, migration, and invasion of HCT116 and HT29 cell lines. DIQ also induced apoptosis and an increase in ROS along with an accumulation of cells in the sub-G1 region. Consistent with the in vitro data, DIQ exhibited reduction in the tumor growth and proliferation in vivo.

Our major focus in this study was to evaluate the ability of DIQ to target CSCs in HCT116 and HT29 cells using a 3D sphere formation assay. CSCs are a rare subpopulation of stem-like tumor cells that are responsible for tumor relapse[28,29]. The increase of SFU in both CRC cell lines from generation 1 up to generation 5 suggests an enrichment in CSCs upon propagation, thus confirming the advantage of using the 3D sphere formation assay. Treatment of HCT116 and HT29 cells with DIQ at a concentration as low as 1 μmol/L targeted the subpopulation of stem/progenitor cells over five generations as reflected by the drastic decrease in the SFU and the sphere size in both cell lines. HT29 spheres were more sensitive to 1 μmol/L DIQ, and an eradication of HT29 spheres occurred at generation 3. The interesting finding of DIQ not affecting non-tumorigenic FHS74Int cells makes DIQ somewhat selective to cancer cells, which is the most essential aspect sought after in anticancer drugs.

To understand what molecular pathways could be targeted by DIQ, we focused mainly on the pathways implicated in CSCs. Multiple signaling systems are involved in resistance of CSCs to therapy. It is widely accepted that the Wnt/β-catenin pathway is the most relevant signaling pathway for colon cancer development. Wnt signaling contributes to stem cell development, tumorigenicity, and oncogenesis[30,31]. This pathway is mechanistically responsible for drug resistance of colon CSCs. Increasing evidence validates that this pathway can interact with other oncogenic signaling pathways, such as those involving MAPK, PI3K, AKT, and ERK, which are aberrantly activated in many human cancers[32,33]. Indeed, evidence has shown that AKT and ERK are overexpressed in human CRC[32].
Figure 8 Establishment and characterization of patient-derived organoids from colon cancer patient 1. A: Representative image of organoids derived from patient 1 stained with hematoxylin and eosin (HE); B: Representative bright-field images of organoids at generation (G)1, G2, and G6. Fresh tumor tissues were enzymatically digested, and single cells were plated in 90% growth factor-reduced Matrigel. G1 organoids were successfully propagated up to G6. Images were visualized by Axiovert inverted microscope at × 5, × 10, and × 20 magnification. Scale bar 100 μm; C: Immunofluorescent images of organoids stained with colon lineage epithelial markers cytokeratin (CK)19 and CK8 and stem cell marker CD44. The nuclei were stained with anti-fade Fluorogel II with 4′,6-diamidino-2-phenylindole (DAPI). Representative confocal microscopy images were acquired using a Zeiss LSM 710 laser scanning confocal microscope. Scale bar 100 μm; D: Representative bright-field images of G2 organoids treated with diiminoquinone (DIQ) (0.5 and 1 μmol/L) or 5-fluorouracil (5FU) (3 μmol/L). Organoid formation count (OFC) and size were calculated, and mean values were reported as mean ± standard error of the mean (\( ^{a}p < 0.05, ^{b}p < 0.01, ^{c}p < 0.001 \)). Images were visualized by Axiovert inverted microscope at × 5 and × 20 magnification. Scale bar 100 μm.


Thus, identifying drugs that target these oncogenic pathways could make a solid rationale for the targeted therapy of cancers. The result of western blot analysis showed that the ratio of both phosphorylated AKT to total AKT (p-AKT/AKT) and phosphorylated ERK to total ERK (p-ERK/ERK), which are key players of AKT/ERK pathways, were decreased upon DIQ treatment in CRC spheres. These findings suggest that DIQ suppressed sphere growth and formation via dual inhibition of AKT/ERK dependent signaling pathways.

We additionally investigated the protein levels of the key stem cell markers in CRC, CD133, and β-catenin, which are involved in chemotherapy resistance. Interestingly, the expression of CD133 and β-catenin were dramatically downregulated after DIQ treatment. Moreover, upon DIQ treatment, there was a significant decrease in the expression of CD44 and CK19 in both CRC cell lines, which were highly expressed in control spheres, along with a decrease in the expression of Ki67 and PCNA. It is interesting to note that CK19, which is considered a tumor marker in CRC, is specifically and stably expressed in primary and metastatic CRC cells. Altogether, this suggests that DIQ could be considered a novel therapeutic compound for suppressing CSC self-renewal.

DIQ-mediated apoptosis and inhibition of cell cycle progression was dependent on the upregulation of p21, which is a known tumor suppressor[34], promotes ROS accumulation, binds to PCNA, and inhibits cell cycle progression[35]. ROS is one of the major inducer of DNA damage. Induction of ROS generation induces increased stress on cancer cells leading to cancer cell death. Interestingly, DIQ treatment induced ROS production in CRC cell lines suggesting that an increase in ROS might also be involved in the anticancer effects of DIQ.
Figure 9 Effect of diiminoquinone on established patient-derived organoids from colon cancer patient 3. A: Immunohistochemistry images of tissue derived from patient 3 stained with hematoxylin and eosin (HE). Immunofluorescent images of tissue stained with colon lineage epithelial markers cytokeratin (CK)19 and stem cell marker CD44. The nuclei were stained with anti-fade Fluorogel II with 4', 6-diamidino-2-phenylindole (DAPI). Representative confocal
microscopy images were acquired using a Zeiss LSM 710 laser scanning confocal microscope. Scale bar 20 μm; B: Immunofluorescent images of organoids derived from colon cancer patient 3 at generation (G)2 in the presence and absence of diiminoquinone (DIQ) treatment (0.5 and 1 μmol/L) stained with colon lineage epithelial markers CK19 and stem cell marker CD44. The nuclei were stained with anti-fade Fluorogel II with DAPI. Representative confocal microscopy images were acquired using a Zeiss LSM 710 laser scanning confocal microscope. Scale bar 20 μm. Representative bright-field images of organoids derived from colon cancer patient 3 at G2 in the presence and absence of DIQ treatment (0.5 and 1 μmol/L). Organoid formation count and size were calculated, and mean values were reported as mean ± standard error of the mean (P < 0.05, P < 0.01, P < 0.001). Images were visualized by Axiovert inverted microscope at ×5 and ×20 magnification. Organoid formation count (OFC) and size of G were calculated, and mean values were reported as mean ± standard error of the mean (P < 0.05, P < 0.01, P < 0.001). Scale bar 100 μm.

Since DIQ-induced apoptosis in HCT116 colonospheres through an increase in TUNEL positivity as we previously reported[22], we assessed whether DNA damage was activated in the spheres derived from both cell lines. We evaluated the expression of γH2AX, which is a DNA double-strand damage biomarker and could be a classical cancer prognostic factor[36,37]. The loss of DNA damage in CRC is involved in the development of therapeutic resistance[37]. In addition, quinones and oxaliplatin have been shown to induce apoptosis of CRC cells by activating DBS and activating γH2AX expression[7,38]. Interestingly, DIQ increased the expression of γH2AX in both CRC cells; clearly emphasizing that DIQ is a potent inducer of DNA damage.

Interestingly, this study also demonstrated effects of DIQ in patient-derived organoids. This model closely recapitulates tissue architecture and cellular composition and is used to assess the self-renewal and differentiation capacities of the organoid CSC, including growth kinetics and drug sensitivity[12,33]. Testing drug efficacy in colon patient-derived organoids holds great promise for personalized medicine and exhibits a significant potential to predict patient response and connect compound screening and clinical trials[39,40]. Since drug resistance to chemotherapy is a serious challenge in treating solid tumors, drug exposure studies on the patient-derived organoids help in choosing specific chemotherapy regimens for patients with malignant disease. Since chemotherapy response in CRC treatment varies between patients, the current study used patient-derived CRC organoids to evaluate the antineoplastic effect of DIQ in targeting stem cells. The established colon organoids expressed the CRC epithelial marker lineage CK19 and the CSC cell marker CD44. This observed co-expression recapitulates the architecture and the characteristics of colon tissues. Notably, DIQ caused a prominent inhibitory effect on the growth of CRC organoids from various patients at different CRC stages, emphasizing its antitumor potential in CRC patients. This effect was either more than or as potent as that of 5FU, emphasizing its inhibitory effect. The results of response of HCT116 and HT29 cell-derived organoids to DIQ treatments were consistent with that of patient-derived organoids. We, therefore, for the first time revealed that DIQ targeted the CSC in patient-derived colon organoids.

The present study has several limitations. The two major limitations in organoid establishment and subsequent applications were the small size of the patient tissue and the availability of tissues at the time of the study. As a clinical study, the patient sample size was relatively small. Additionally, the percentage success rate of deriving colon patient derived organoids was not more than 42%; only 5 out of 12 specimens were successfully established as colon organoids. This could possibly be due to limitations in tissue quality as well. A larger cohort is still required to further investigate and evaluate the effect of DIQ in translational medicine.

**CONCLUSION**

In conclusion, we demonstrated for the first time that DIQ reduces self-renewal capacity of colorectal tumors and prevents therapy resistance in patient-derived organoids through interfering with the stem cell Wnt/-catenin and AKT and ERK pathways that are involved in CRC tumorigenesis. Also, the effect of DIQ was involved in the major cell fate responses including apoptosis, cell cycle arrest, and stress response. DIQ inhibits the key processes of CRC tumorigenesis, including cell growth, proliferation, migration, and invasion. Our findings strongly suggest that DIQ could be a promising compound for treatment of CRC patients and could be clinically used as a non-toxic compound for targeting human colon cancer stem/progenitor cells.
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A

Control  |  DIQ 0.5 μmol/L  |  DIQ 1 μmol/L  |  5FU 3 μmol/L

Patient 2

![Images of cells at 5x and 20x magnification with diameters labeled in μm]

Graphs showing OPC and organoids diameter for Patient 2:

- **OPC**
  - Control vs. DIQ 0.5 μmol/L: Significant difference labeled as 'b'
  - Control vs. DIQ 1 μmol/L: Significant difference labeled as 'b'
  - Control vs. 5FU 3 μmol/L: No significant difference

- **Organoids diameter**
  - Control vs. DIQ 0.5 μmol/L: Significant difference labeled as 'c'
  - Control vs. DIQ 1 μmol/L: No significant difference
  - Control vs. 5FU 3 μmol/L: Significant difference labeled as 'c'

B

Control  |  DIQ 0.5 μmol/L  |  DIQ 1 μmol/L  |  5FU 3 μmol/L

Patient 4

![Images of cells at 5x and 20x magnification with diameters labeled in μm]

Graphs showing OPC and organoids diameter for Patient 4:

- **OPC**
  - Control vs. DIQ 0.5 μmol/L: Significant difference labeled as 'c'
  - Control vs. DIQ 1 μmol/L: Significant difference labeled as 'c'
  - Control vs. 5FU 3 μmol/L: Significant difference labeled as 'c'

- **Organoids diameter**
  - Control vs. DIQ 0.5 μmol/L: Significant difference labeled as 'c'
  - Control vs. DIQ 1 μmol/L: Significant difference labeled as 'c'
  - Control vs. 5FU 3 μmol/L: Significant difference labeled as 'c'
Figure 10 Diiminoquinone reduced the growth of the patient-derived organoids from different colon cancer patients. A: Representative bright field images of generation (G)1 organoids derived from patient 2 (grade 2; stage T3) grown with or without diiminoquinone (DIQ) or 5-fluorouracil (5FU). Organoid formation count (OFC) was calculated in duplicate wells per condition. The quantification of the average diameter was calculated. The data of OFC and size are presented in two separate graphs; B: Representative bright field images of G4 organoids derived from patient 4 (grade 2; stage T2) grown with or without DIQ or 5FU. OFC was calculated in duplicate wells per condition. The quantification of the average diameter size was calculated. The data of OFC and size are presented in two separate graphs; C: Representative bright field images of G2 organoids derived from patient 5 (grade 2; stage T3) grown with or without DIQ or 5FU. OFC was calculated in duplicate wells per condition. The quantification of the average diameter was calculated. The average mean of OFC and size are presented in two separate graphs. All mean values were reported as mean ± standard error of the mean (aP < 0.05, bP < 0.01, cP < 0.001). Scale bar, 100 μm.

**ARTICLE HIGHLIGHTS**

**Research background**
Colorectal cancer (CRC) is a multistep genetic disorder caused by sequential mutational events in signal transduction pathways occurring along with progression of the cancer. Quinone containing compounds have been reported as one of the promising novel anticancer therapeutics against CRC. However, the effects of diiminoquinone (DIQ) on CRC stem cells have not been extensively investigated yet.

**Research motivation**
To explore the promising anticancer effects of a novel quinone, DIQ, on CRC.

**Research objectives**
To investigate the anticancer potential of novel therapeutic DIQ on CRC using two-dimensional and three-dimensional models.

**Research methods**
MTT and trypan blue exclusion assays were employed to assess the anti-proliferative effect of DIQ on HCT116 and HT29 cell lines in vitro. Propidium iodide DNA and dihydroethidium staining were performed to determine cell cycle distribution and reactive oxygen species production in response to DIQ, respectively. Wound healing and transwell invasion assays were used to determine the invasion and migration ability of DIQ, respectively. Then, a sphere formation model was used to evaluate the potency of DIQ on targeting cancer stem cells in CRC cells for up to five generations. Immunofluor-
escent analysis and western blot were performed to elucidate the mechanism of action of DIQ in CRC. Organoid model was used to assess DIQ response on established organoids from fresh colorectal tissue samples from consenting patients.

**Research results**
DIQ reduced the self-renewal capacity of CRC cells and targeted the growth of colon cancer patient-derived organoids. DIQ downregulated the expression of key markers involved in the oncogenic stem cell Wnt/-catenin, AKT, and ERK signaling pathways that are involved in CRC tumorigenesis. Also, DIQ decreased proliferation, migration, and invasion and induced apoptosis, cell-cycle arrest, and reactive oxygen species.

**Research conclusions**
Our findings strongly suggest that DIQ could be a promising novel therapeutic for the treatment of CRC patients. This study represents the first documentation of the molecular mechanism of the novel anticancer therapeutics DIQ via targeting cancer stem cells, findings that have potential therapeutic implications for colon cancer patients.

**Research perspectives**
Further research on the DIQ mechanisms that are involved in CRC tumorigenesis is needed to be performed in the future. A larger cohort is still required to further investigate and evaluate the effects of DIQ in translational medicine.

**ACKNOWLEDGEMENTS**
We are thankful to all members of the Gali-Muhtasib and Abou-Kheir Laboratory and the staff of the core facilities in the DTS Building at the American University of Beirut for their technical help and support.

**FOOTNOTES**

**Author contributions:** Monzer A carried out lab work as part of her PhD thesis, wrote the manuscript, and performed data analysis and interpretation of data (e.g., biostatistics, statistical analysis, and editing); Wakimian K, Ballout F, Al Bitar S, and Yehya A performed initial lab work and participated in data collection; Faraj W, Tawil A, Doughan S, Hussein M, Kanso M, and Saheb N helped in clinical data curation and the consent form for colon cancer patient samples at the American University of Beirut Medical Center; Gali-Muhtasib H and Abou-Kheir W conceived the project, supervised the work, and edited the manuscript draft; All authors have reviewed and approved the final manuscript.

**Institutional review board statement:** All specimens from the patients were obtained after their informed consent. All experiments involving human subjects were performed in agreement with all ethical considerations of the Institutional Review Board.

**Institutional animal care and use committee statement:** Prior to any mouse experiment, all mice protocols were reviewed and approved by the Institutional Animal Care and Use Committee (American University of Beirut, Institutional Animal Care and Use Committee).

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The ARRIVE Guidelines have been adopted. The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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Case Control Study

Previous hepatitis B viral infection—an underestimated cause of pancreatic cancer

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Pham TTT, Viet Nam; Sultana C, Romania; Tai DI, Taiwan

Received: April 20, 2022
Peer-review started: April 20, 2022
First decision: June 2, 2022
Revised: June 15, 2022
Accepted: August 16, 2022
Article in press: August 16, 2022
Published online: September 7, 2022

Abstract

BACKGROUND
The etiology of pancreatic cancer remains unclear. This limits the possibility of prevention and effective treatment. Hepatitis B virus (HBV) is responsible for the development of different types of cancer, but its role in pancreatic cancer is still being discussed.

AIM
To assess the prevalence of previous HBV infection and to identify viral biomarkers in patients with pancreatic ductal adenocarcinoma (PDAC) to support the role of the virus in etiology of this cancer.
METHODS
The data of 130 hepatitis B surface antigen-negative subjects were available for the final analysis, including 60 patients with PDAC confirmed by cytology or histology and 70 sex- and age-matched controls. All the participants were tested for HBV biomarkers in blood [antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis B surface antigen (anti-HBs) and HBV DNA], and for those with PDAC, biomarkers in resected pancreatic tissues were tested (HBV DNA, HBV pregenomic RNA and covalently closed circular DNA). We performed immunohistochemistry staining of pancreatic tissues for hepatitis B virus X antigen and Ki-67 protein. Non-parametric statistics were used for the analysis.

RESULTS
Anti-HBc was detected in 18/60 (30%) patients with PDAC and in 9/70 (13%) participants in the control group ($P = 0.029$). Accordingly, the odds of PDAC in anti-HBc-positive subjects were higher compared to those with no previous HBV infection (odds ratio: 2.905, 95% confidence interval: 1.191-7.084, standard error 0.455). HBV DNA was detected in 8 cases of PDAC and in 6 of them in the pancreatic tumor tissue samples only (all patients were anti-HBc positive). Blood HBV DNA was negative in all subjects of the control group with positive results of the serum anti-HBc test. Among 9 patients with PDAC, 5 revealed signs of replicative competence of the virus (covalently closed circular DNA with or without pregenomic RNA) in the pancreatic tumor tissue samples. Hepatitis B virus X antigen expression and active cell proliferation was revealed by immunohistochemistry in 4 patients with PDAC in the pancreatic tumor tissue samples.

CONCLUSION
We found significantly higher risks of PDAC in anti-HBc-positive patients. Detection of viral replication and hepatitis B virus X protein expression in the tumor tissue prove involvement of HBV infection in pancreatic cancer development.

Key Words: Hepatitis B virus; Previous hepatitis B; Occult hepatitis B virus infection; Pancreatic cancer; Pancreatic ductal adenocarcinoma

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Core Tip: Hepatitis B viral (HBV) infection is responsible for different types of cancer. Its role in pancreatic ductal adenocarcinoma (PDAC) remains unclear. This study assessed the prevalence of previous HBV infection and to identify viral biomarkers in patients with PDAC to support the role of the virus in the etiology of this cancer. Anti-HBc-positive subjects had an almost 3-fold greater chance of PDAC compared to the controls. Detection of viral replication and hepatitis B virus X protein expression in the tumor tissue show a possible involvement of HBV infection in pancreatic cancer development. Previous HBV infection is currently an underestimated cause of PDAC.

Citation: Batskikh S, Morozov S, Dorofeev A, Borunova Z, Kostyushev D, Brezgin S, Kostyusheva A, Chulanov V. Previous hepatitis B viral infection–an underestimated cause of pancreatic cancer. World J Gastroenterol 2022; 28(33): 4812-4822
URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4812.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4812

INTRODUCTION
Pancreatic cancer (PC) is an aggressive gastrointestinal malignancy with a low rate of early detection, poor survival and a limited number of therapeutic options. It causes more than 430000 deaths yearly worldwide[1]. This makes PC the third leading cause of cancer-related deaths in the United States and the fourth in the European Union[2,3]. The incidence rate of PC is growing, while the improvement in the survival rates is negligible[4]. Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent type of PC and is found in 85% of cases[5]. The etiology of PC remains unclear, which limits the possibility for prevention and effective treatment. Early detection of PC remains a challenge. Therefore, it is still relevant to explore etiological factors of PDAC further and to identify subjects at risk of the disease. Numerous risk factors for the disease have been identified (smoking, excessive alcohol intake, history of chronic pancreatitis, obesity and diabetes, etc)[6]. Some viruses, including hepatitis B virus (HBV), are responsible for the development of different types of cancer, but their role in PC is still being discussed.
In endemic regions, like Southeast Asia, the blood markers of current HBV infection are commonly found in subjects with PC. Some authors from endemic regions report that HBV infection is not associated with the risk of developing PC after adjusting for age, sex, diabetes and smoking[9].

Cohort studies from Northern Europe (Denmark, Sweden), where HBV infection is not widespread, showed conflicting results and made an association between PC and HBV infection questionable[10-12]. In most of these studies, association of PC with previous HBV infection (PBI) was not considered. However, it may be important as the risk of liver cancer development perseveres even after hepatitis B surface antigen (HBsAg) loss[13-15].

Cancerogenic mechanisms of HBV infection may be explained by the integration of viral DNA fragments into the genome of host cells or persistence of the viral genome as a covalently closed circular DNA (cccDNA), which plays the role of viral reservoir and template for life-long synthesis of new virions. Both are responsible for preserved expression of viral proteins (especially HBx), which can lead to potentially oncogenic mutations[16-18].

Thus, not only active hepatitis B, but also previous HBV infection may contribute to PC development. Detection of HBV DNA and viral antigens in the pancreatic tumor tissues may provide direct evidence of the involvement of the virus in the etiology of this cancer. However, only a few studies demonstrated the presence of HBV DNA (cccDNA) and/or viral antigens in pancreatic tumor tissue. Therefore, the aim of our study was to assess the prevalence of PBI and to identify viral biomarkers in patients with PDAC to support the role of the virus in the etiology of this cancer.

MATERIALS AND METHODS

Study population
The study was based on the data of complex examination of patients that applied for PC treatment to Moscow Clinical Scientific Center named after A.S. Loginov from January 2019 to November 2020. Subjects of the control group were also recruited. The study (registered AAAA-A18-118021590196-1, AAAA-A20-12005190006-1 at www.rosrid.ru) was approved by the Local Ethics Committee and was conducted in accordance with the Declaration of Helsinki (1968) and its consequent revisions. All subjects signed a written informed consent form before the enrollment.

Inclusion criteria
Patients of both sexes, older than 18 years, willing to participate in the study were eligible.

In the group of PC, we enrolled patients with histologically or cytologically confirmed PDAC. In the control group we enrolled generally healthy subjects who applied for routine check-ups or treatment of other non-malignant gastrointestinal conditions and whose data of abdominal ultrasound and/or computed tomography revealed no signs of focal lesions in the pancreas.

Exclusion criteria
Other/indeterminate types of PC or non-malignant lesions beside PDAC (for the main group); positive blood test for HBsAg, hepatitis C virus or HIV antibodies; past surgery for PC; current or previous treatment with interferons, nucleos(t)ide analogues for HBV infection or other reasons; clinically significant diseases or health disorders, making it impossible to perform procedures required by the study protocol.

Confirmation of the conditions of interest
PBI was defined as the presence of antibody to hepatitis B core antigen (anti-HBc) with or without antibody to hepatitis B surface antigen (anti-HBs) or HBV DNA in serum[19]. Control subjects were matched for age (within 2 years), sex and race/ethnicity with the PDAC patients. Study design is shown in Figure 1.

Study procedures
To exclude health conditions able to affect results of the study, all patients underwent routine diagnostic procedures (including but not limited to blood tests, electrocardiogram, abdominal ultrasound and chest X-ray) within standards of care.

All the participants were tested for HBV biomarkers in blood (HBsAg, anti-HBc, anti-HBs). Those HBsAg-negative with positive anti-HBc result were tested for HBV DNA in blood. Anti-HBc-positive patients with PDAC were examined for HBV DNA in the pancreatic tumor tissue.

Tumor tissues of anti-HBc-positive patients with PDAC underwent examination for HBV biomarkers (HBV pregenomic RNA and cccDNA) and immunohistochemistry staining for hepatitis B virus X antigen (HBxAg) and Ki-67 protein in cases of the signed informed consent for these tests and sufficient quantity and good quality of the samples.
All anti-HBc-positive participants were tested for the presence of HBV DNA in the blood. In addition, all 18 anti-HBc-positive patients with PDAC were tested for the presence of HBV DNA in pancreatic tumor tissue. In 8 of them, the quality and quantity of samples were suitable for additional testing for HBV pregenomic RNA and cccDNA. Five patients had eligible samples according to these criteria and gave additional consent for immunohistochemical staining for HBxAg and Ki-67 protein.

**Collection of samples:** Blood samples were taken after overnight fasting, coded and processed immediately at the local laboratory according to the standard instructions. Pancreatic tumor tissue samples were obtained during surgery or diagnostic biopsy, coded and processed locally. They were stained with hematoxylin-eosin and assessed by a qualified morphologist.

**Immunology:** Serum samples were tested for HBsAg, anti-HBc IgG, anti-HBs, hepatitis C virus and HIV antibodies. These tests were performed with the use of a Sunrise analyzer (Tecan GmbH, Austria) and specific immunoassays kits (Vector-Best Co., Russia).

**Analysis of HBV nucleic acids:** Plasma HBV DNA was isolated using commercial AmpliSens Riboprep kit (AmpliSens Biotechnologies, Russia) according to manufacturer’s instructions and quantified using the PCR assay AmpliSens HBV-FL (AmpliSens Biotechnologies) kit (lower limit of detection of 10 IU/mL).

To isolate nucleic acids from biopsies, samples were first homogenized in the MagNA Lyser (Roche Diagnostics, Switzerland). HBV DNA was isolated by AmpliSens Riboprep kit (AmpliSens Biotechnologies) and quantified by AmpliSens HBV-FL (AmpliSens Biotechnologies) kit.

To quantify cccDNA, nucleic acids were first treated with T5 exonuclease (New England Biolabs, United Kingdom) at 37 °C for 60 min and inactivation at 70 °C for 20 min[20]. HBV cccDNA was quantified with specific sets of primers and probes and normalized to genomic β-globin.

Specific sets of primers (Table 1) and TaqMan fluorescent probes were used for PCR analysis to detect HBV DNA in pancreatic tissue samples.
Table 1 List of specific sets of primers and probes used for the analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cccDNA</td>
<td>Fw: CCGTGTCACCATCGGTTCAC</td>
</tr>
<tr>
<td></td>
<td>Rev: GCACAGCTTGAGGCTTGA</td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-CATGGAGACCACCGTGAACCC-BHQ1</td>
</tr>
<tr>
<td>β-globin</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V31-FEP-CE (AmpliSens)</td>
</tr>
<tr>
<td></td>
<td>HPV HCR-Screen (AmpliSens)</td>
</tr>
</tbody>
</table>

Table 1: List of specific sets of primers and probes used for the analysis. cccDNA: Covalently closed circular DNA; Fw: Forward; Rev: Reverse; HPV: Human papillomavirus; HCR: High carcinogenic risk; AmpliSens: AmpliSens Biotechnologies, Russia.

To analyze pregenomic HBV RNA (pgRNA HBV), nucleic acids were treated with RNase-free DNase I (NEB) for 30 min at 37 °C, purified by using AmpliSens Riboprep kit (AmpliSens Biotechnologies), reverse transcribed by AmpliSens Reverta-FL (AmpliSens Biotechnologies) and quantified by AmpliSens HBV-FL (AmpliSens Biotechnologies) kit. CFX96 Real-Time System (Bio-Rad, United States) PCR machine was used for the analysis of plasma and pancreatic tissue samples.

Immunohistochemistry of pancreatic tissues was performed after deparaffinization. Slides were fixed in 4% paraformaldehyde, washed three times in Tris-HCl (50 mM, pH 8.0) followed by incubation with a blocking buffer (0.02% of Triton X-100, 10% horse serum, and 150 mmol/L NaCl in Tris-HCl, 50 mmol/L, pH 8.0) for 30 min and 1 h staining with primary rabbit anti-HBx (ab39716) (Abcam, United Kingdom). Then, slides were washed three times for 5 min in a washing buffer (0.02% of Triton X-100 and 200 mmol/L NaCl in Tris-HCl, 50 mmol/L, pH 8.0) and incubated for 1 h with secondary Alexa Fluor 594 goat anti-rabbit antibodies (ab150080) (Abcam). After that, the slides were treated with primary tagged Alexa Fluor® 488 rabbit anti-Ki-67 and Hoechst 33342 (ab228551) for 1 h, washed three times for 5 min in washing buffer and finally mounted with a Fluoroshield reagent (Abcam). Images were captured using Thunder imaging systems (Leica Microsystems, Germany) with 10 × objectives. Ki-67 and HBxAg staining was analyzed using LAS X (Leica Microsystems). Ki-67 index was counted as the percentage of Ki-67-positive cells[21].

**Statistical analysis**

Statistica 12.0 software (StatSoft Inc., United States) was used for analysis of the data. Statistical processing of the obtained data was carried out using nonparametric statistics. Quantitative indicators were preliminarily assessed for compliance with the normal distribution using the Kolmogorov-Smirnov and Lilliefors tests. When quantitative indicators’ distributions differed from normal, we used medians and the interquartile ranges (25%-75%) for the description and processed the data using the Mann-Whitney U test. Nominal data were compared using Pearson χ² test with Yates’s correction. P values < 0.05 were considered significant. Odds ratio (OR) and 95% confidence interval (95%CI) calculations were performed to assess the association between PDAC and PBI marker detection.

**RESULTS**

Data of 60 patients with PDAC and 70 participants of the control group were available for the final analysis. Demographic and viral characteristics of the participants are shown in Table 2.

In patients with PDAC, anti-HBc antibodies were found more often than in the control group ($P = 0.029$). Accordingly, the odds of PDAC in anti-HBc-positive subjects were significantly higher compared to those who had no PBI (OR: 2.905, 95%CI: 1.191-7.084, standard error 0.455).

Overall, HBV DNA was found in 8 anti-HBc-positive patients with PDAC. In 2 subjects it was detected in both the blood and pancreatic tumor samples, whereas in the other 6 participants the testing gave positive results only in the pancreatic tumor tissues. No positive results for HBV DNA were obtained in the control group.

The markers confirming replicative competence of HBV (cccDNA with or without pgRNA) were found in the pancreatic tumor tissue samples in 5 patients with PDAC. In 1 subject with a positive test on HBV DNA in the pancreatic tissue examination on cccDNA and pgRNA was not performed.

In those with detectable HBV DNA, viral load in the pancreatic tissue was 632 (390-851) IU/mL [median (25%-75%)].

HBxAg expression and active cells’ proliferation was revealed by immunohistochemistry in 4 participants with PDAC in the pancreatic tumor tissue samples (Table 3 and Figure 2). The number of HBx-expressing cells in them did not exceed 10%.
Ki-67 proliferative index in subjects with PDAC in the cohort of special examination was 79.1 (45.2–86.4) [median (25%-75%)]. All HBx-expressing cells were also Ki-67 positive.

**DISCUSSION**

The results of the present study demonstrate the association of PBI with PDAC and provide direct molecular evidence for the presence of HBV biomarkers in the pancreatic tumor tissue. In 8 of our patients with PDAC, HBV DNA was detected in the pancreatic tumor tissue. In 5 of them, replicative competence of HBV DNA in the pancreas was supported by detection of cccDNA (with or without pgRNA). Identification of cccDNA and pgRNA (transcribed only from cccDNA) additionally suggests that these patients saved a silent replication of the virus in the pancreatic tissue. Detection of the virus nucleic acids in pancreatic tissue only (with no HBV DNA present in blood) in most of subjects excludes...
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Figure 2 Immunohistochemistry of resected pancreatic tumor tissues (obtained during surgery). Case: Anti-HBc-positive patient with pancreatic ductal adenocarcinoma. Control: Patient with pancreatic ductal adenocarcinoma, who was negative for hepatitis B virus biomarkers. A: Images at magnification × 10; B: Images at magnification × 100. Samples were stained for Ki-67 protein (green fluorescence) and X antigen of hepatitis B virus (HBxAg) (red fluorescence). Cell nuclei were counterstained by Hoechst33342 dye (blue). Asterisks indicate HBxAg/Ki-67 co-stained cells.

DOI: 10.3748/wjg.v28.i33.4812 Copyright ©The Author(s) 2022.

Viral infections, including those caused by HBV were recognized among the modifiable risk factors of PC development[22,23]. The data of a meta-analysis of case-control and observational studies (number of subjects with PC: 5883) showed that the odds of PC were significantly higher in chronic or inactive HBsAg carriers [OR: 1.60 (95%CI: 1.26-2.05)] and anti-HBc-positive but anti-HBs-negative individuals [OR: 1.76 (95%CI: 1.05-2.93)] compared to those who were never exposed to HBV infection[7,24]. In our study, the odds of PDAC were even higher, and anti-HBc-positive subjects had an almost 3-fold greater chance of PDAC compared to the controls.

Only a few studies have demonstrated molecular evidence of possible HBV involvement in pancreatic tumor development by identifying HBV DNA (cccDNA) and/or its antigens in pancreatic tumor tissue, and only a limited number of subjects (especially HBsAg-negative but anti-HBc-positive) were involved[25,26]. Although certain pathogenetic mechanisms of PC associated with hepatitis B infection need to be explained in specially planned studies, some assumptions could be made.

HBV is a known carcinogen and is one of the main causes of hepatocellular carcinoma in endemic regions[8]. However, in HBV endemic areas, such as Africa and East Asia, there is a relatively low rate of PC-related deaths. Probably due to high mortality from other causes (including HBV-associated hepatocellular carcinoma), there is not enough time for the development of PC in people with PBI.

HBV integrates into the genome of infected cells, causes genomic aberrations, enhances expression of oncogenes or inhibits tumor suppressors and leads to the cancer development[14]. Similar mechanisms are possible in non-liver carcinogenesis, including the pancreas[25,27]. Pancreatic beta cells and hepatocytes develop from the ventral foregut endoderm during ontogenesis and thus may share characteristics that are favorable for HBV replication and virus-induced tumor development[28]. It seems that malignant transformation in the pancreas is not provoked by direct cellular damage and is caused by the integration of HBV DNA into the genome of pancreatic cells and subsequent disruption of the functions of anti-oncogenes or by stimulation of pro-oncogenes' activity[29]. The replication of HBV in pancreatic tissue may decrease with time. However, DNA fragments of the virus integrated into the genome of host cells continue to express viral proteins (especially regulatory protein X) of HBV responsible for carcinogenesis. The expression capability of HBx from integrated fragments of the viral genome in tumor tissues when replication is absent was confirmed in hepatocellular carcinoma[30-32].
In our study, immunohistochemistry revealed expression of HBx in the pancreatic tumor tissue in 4 out of 5 HBsAg-negative and anti-HBc-positive patients with PDAC. Replicative competence of HBV (detected cccDNA) was found in 3 of them. This may mean that in 1 patient, expression of HBx was caused only by the integration of the virus into the genome of pancreatic cells. These fragments of viral DNA, which preserve the open reading frame and express HBx, may serve as a basis for carcinogenesis in subjects with PDAC. Although this mechanism may play a role in primary cancer development, its role in PC recurrence is not clear, and further studies are necessary. The low-grade replication may also play a role in HBV reactivation, especially in cases when immunodepressants are used. However, this question is insufficiently studied.

It is not clear whether the number of HBxAg expressing cells is important for cancer development. In hepatocellular carcinoma of HBsAg-negative HBV DNA-positive subjects, the relative number of HBxAg-expressing cells is about 30% within the tumor tissue and 20% in the rest of the liver tissue\[33\]. Similar data for PC are lacking. According to our results, the number of cells producing HBxAg in PDAC is about 4%. It seems that the number of cells producing HBx protein is less important than their presence, at least for PC development. This may be indirectly confirmed by the fact that in all HBxAg-positive subjects in our study proliferative index Ki-67 was significantly higher than 50%, whereas similarly high values of this marker were only found in about 12% of subjects with PDAC\[21,34\].

Detection of cccDNA in pancreatic tissue in HBsAg-negative subject supports the need for revision of the statements of the Taormina Workshop (2018), which defines occult HBV infection as the presence of replication-competent HBV DNA (i.e. cccDNA in the liver and/or HBV DNA in the blood of people who test negative for HBsAg by currently available assays)\[16\]. As extrahepatic HBV replication may occur in HBsAg-negative subjects (which was confirmed in the course of our study), it is reasonable not to indicate in the statement the specific organ for HBV DNA (cccDNA) detection.

Involvement of PBI in PC development requires revision of the ultimate targets of antiviral treatment. “Sterilizing cure” (undetectable HBsAg in blood in combination with the absence of DNA HBV in any tissues, including cccDNA and integrated viral DNA) was recognized unachievable in the near future \[35\]. However, “functional cure” (defined as sustained clearance of HBs with or without HBs-seroconversion and non-detectable HBV DNA in blood after the course of treatment) evidently cannot affect the expression of oncogenic proteins of HBV (especially HBxs) and thus diminish the chances of cancer development. Although it is impossible to achieve eradication of cccDNA and integrated fragments of the viral genome with currently existing means, this should be stated as the ultimate goal for future therapy options.

The limitation of the study is a relatively small number of patients. Moreover, examination of the tumor tissues on cccDNA, pgRNA and HBxAg was possible for only a few of the 18 anti-HBc-positive subjects with PDAC due to the quality of the obtained specimens. Further randomized multicenter studies are necessary to confirm the obtained results, prove the role of HBV infection in the etiology of PC and clarify carcinogenic mechanisms in them.

**CONCLUSION**

An almost three-fold risk of PDAC was found in HBsAg-negative but anti-HBc-positive subjects. Detection of silent viral replication and pro-oncogenic HBx protein expression in the tumor tissue suggest involvement of HBV infection in PC development. PBI seems to be an underestimated cause of PDAC at the moment.

**ARTICLE HIGHLIGHTS**

**Research background**

The etiology of pancreatic cancer is unclear. This limits possibilities for its prevention and effective treatment. Hepatitis B virus (HBV) is responsible for the development of hepatocellular carcinoma and different types of extrahepatic cancer, but its role in the etiology of pancreatic cancer is still being discussed.

**Research motivation**

The epidemiological relationship of previous HBV infection (PBI) with pancreatic cancer and identification of viral biomarkers within the tumor tissue may provide support for this. However, there is still a lack of such reports, especially from non-endemic regions for HBV infection.

**Research objectives**

In our study, we aimed to assess the prevalence of PBI and to identify viral biomarkers in patients with pancreatic ductal adenocarcinoma (PDAC) to support the role of the virus in the etiology of this cancer.
Research methods
The data of 130 hepatitis B surface antigen-negative subjects were included in the final analysis (60 patients with PDAC confirmed by cytology or histology and 70 sex- and age-matched controls). All the participants were tested for HBV biomarkers in blood (antibody to hepatitis B core antigen, antibody to hepatitis B surface antigen and HBV DNA). Those with PDAC were tested for biomarkers in resected pancreatic tissues [HBV DNA, HBV pregenomic RNA and covalently closed circular DNA (cccDNA)]. Additionally, we performed immunohistochemistry staining of pancreatic tissues for hepatitis B virus X antigen and Ki-67 protein. Non-parametric statistics were used for the analysis.

Research results
We have established that 18/60 (30%) patients with PDAC and 9/70 (13%) participants in the control group ($P = 0.029$) were anti-hepatitis B core antigen-positive. HBV DNA was detected in 8 anti-hepatitis B core antigen-positive patients of PDAC (in 6 of them—in the pancreatic tumor tissue samples only) but in neither subjects of the control group. In 5 patients with PDAC we revealed signs of replicative competence of the virus (cccDNA with or without pregenomic RNA) in the pancreatic tumor tissue samples. Hepatitis B virus X antigen expression and active cells’ proliferation was revealed by immunohistochemistry in 4 participants with PDAC in the pancreatic tumor tissue samples.

Research conclusions
PBI seems to be an underestimated cause of PDAC.

Research perspectives
Larger studies are necessary to assess risks of PDAC in subjects with PBI and define HBV-associated mechanisms of carcinogenesis in them.

ACKNOWLEDGEMENTS
The authors acknowledge all study participants.

FOOTNOTES

Author contributions: Batskikh S and Morozov S designed this study; Batskikh S collected and analyzed the data; Borunova Z, Dorofeev A, Kostyushev D, Brezgin S, Kostyusheva A and Chulanov V performed laboratory analyses; Batskikh S, Morozov S and Kostyushev D prepared the figures; Batskikh S performed statistical analysis; Morozov S and Batskikh S drafted the manuscript; All authors critically revised the manuscript and approved its final version.

Supported by Ministry of Science and Higher Education of Russian Federation, No. FGMF-2022-0005; Russian Science Foundation, No. 20-15-00373; and Moscow Healthcare Department, No. AAAA-A18-118021590196-1.

Institutional review board statement: The study was reviewed and approved by the Local Ethics Committee of Moscow Clinical Research Center, No. 9/2016, dated 12DEC2016 and was conducted in accordance with the Declaration of Helsinki (1968) and its consequent revisions.

Informed consent statement: All subjects signed a written informed consent form before the enrollment.

Conflict-of-interest statement: Dr. Batskikh reports grants from Moscow Department of Health during the conduct of the study; personal fees from ABBVIE, personal fees from MSD, personal fees from R-PHARM, outside the submitted work. Dr. Morozov reports grants from Ministry of Science and Higher Education of Russia, during the conduct of the study; personal fees from AstraZeneca, personal fees from Dr. Falk, personal fees from AlfaSigma, grants from Russian Science Foundation, personal fees from Takeda, outside the submitted work. Dr. Chulanov reports grants from Russian Foundation for Basic Research, grants from Russian Foundation for Basic Research, outside the submitted work. Dr. Kostyushev, Dr. Kostyusheva and Dr. Brezgin report grants from Russian Foundation for Basic Research, grants from Russian Foundation for Basic Research, during the conduct of the study. Dr. Dorofeev and Dr. Borunova have nothing to disclose.

Data sharing statement: No additional data are available.

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

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**S-Editor:** Fan JR  
**L-Editor:** Filipodia  
**P-Editor:** Fan JR

**REFERENCES**


Previous HBV infection in PDAC development


Effectiveness, safety, and drug sustainability of biologics in elderly patients with inflammatory bowel disease: A retrospective study

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BACKGROUND
Biologic therapy resulted in a significant positive impact on the management of inflammatory bowel disease (IBD) however data on the efficacy and side effects of these therapies in the elderly is scant.

AIM
To evaluate retrospectively the drug sustainability, effectiveness, and safety of the biologic therapies in the elderly IBD population.

METHODS
Consecutive elderly (≥ 60 years old) IBD patients, treated with biologics [infliximab, etanercept, adalimumab, vedolizumab] were included in the study. The primary endpoint was drug sustainability, defined as the time from the initiation of therapy to discontinuation due to any reason. The secondary endpoints were clinical remission, drug retention, and side effects.

RESULTS
A total of 100 patients were enrolled, with a mean age of 72 years. The median duration of therapy was 24 months. The overall drug sustainability rate was 75%, with a median duration of 36 months. Clinical remission was achieved in 82% of cases, with 90% drug retention. The most common side effect was injection site irritation (10%) and infections (5%).

CONCLUSION
Biologic therapies are effective and safe in elderly patients with IBD, with a good drug sustainability rate. However, further studies are needed to assess the long-term effects and cost-benefit analysis of biologic therapies in this population.

Abstract

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CONCLUSION
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mab (IFX), adalimumab (ADAL), vedolizumab (VDZ), ustekinumab (UST)] followed at the McGill University Inflammatory Bowel Diseases Center were included between January 2000 and 2020. Efficacy was measured by clinical scores at 3, 6-9 and 12-18 mo after initiation of the biologic therapy. Patients completing induction therapy were included. Adverse events (AEs) or serious AE were collected during and within three months of stopping of the biologic therapy.

RESULTS
We identified a total of 147 elderly patients with IBD treated with biologicals during the study period, including 109 with Crohn’s disease and 38 with ulcerative colitis. Patients received the following biologicals: IFX (28.5%), ADAL (38.7%), VDZ (15.6%), UST (17%). The mean duration of biologic treatment was 157.5 (SD = 148) wk. Parallel steroid therapy was given in 34% at baseline, 19% at 3 mo, 16.3% at 6-9 mo and 6.5% at 12-18 mo. The remission rates at 3, 6-9 and 12-18 mo were not significantly different among biological therapies. Kaplan-Meyer analysis did not show statistical difference for drug sustainability (P = 0.195), time to adverse event (P = 0.158) or infection rates (P = 0.973) between the four biologics studied. The most common AEs that led to drug discontinuation were loss of response, infusion/injection reaction and infection.

CONCLUSION
Current biologics were not different regarding drug sustainability, effectiveness, and safety in the elderly IBD population. Therefore, we are not able to suggest a preferred sequencing order among biologicals.

Key Words: Inflammatory bowel disease; Biologics; Elderly; Efficacy; Safety; Adverse events

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Core Tip: Data on the efficacy and side effects of biologic therapies in the elderly inflammatory bowel disease (IBD) population is scant. Our single center study evaluates retrospectively the drug sustainability, effectiveness, and safety of approved biologic therapies in this sensitive population. The major finding of our study was that the drug sustainability and safety of the different biologicals were not significantly different in a large real-world, elderly IBD cohort treated in this single tertiary IBD center. As a consequence, we are still not able to suggest a preferred sequencing order among biologicals.

URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4823.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4823

INTRODUCTION
Crohn’s disease (CD) and ulcerative colitis (UC) are chronic immune-mediated diseases classified as inflammatory bowel diseases (IBD), which can result in progressive bowel damage and disability. Although more common in young adults, the prevalence and the incidence of IBD are increasing in the elderly[1]. Approximately 10%-15% of new IBD diagnoses occur in individuals over 60 years (elderly-onset IBD), while the majority of older patients with IBD are classified as adult-onset IBD, meaning they were diagnosed with IBD between 18-59 years old[2-4]. There has been a revolution in the medical therapy of IBD with the advent of biological agents in the past two decades leading to improved clinical outcomes. Biological therapies were also associated with adverse events (AEs), including infusion/injection reactions, infections, and malignancies. Elderly IBD patients are in many ways a difficult-to-treat patient population, and may be even more vulnerable to AEs due to advanced age, comorbidities and polypharmacy[5].

The proportion of elderly IBD patients in randomized clinical trials (RCTs) is usually small, therefore there is lack of data concerning effectiveness and safety profiles of biologic therapies in this population. This relative paucity of data together with the higher frequency of comorbidities, polypharmacy, and the perceived toxicity of IBD drug therapies in the elderly patients may likely explain the underuse of biological agents and the reported higher rates of steroid use. As reported by studies from France, Sweden and Hong Kong, only 1%-3% of elderly IBD patients received biologic therapy within five to ten
years of follow-up[6-8].

The expected rate of AE and medication interactions may significantly influence the choice of therapy in the elderly IBD population. In a study from Leuven, advanced age was associated with higher rates of serious AEs (SAEs) on anti-tumor necrosis factor (TNF) therapy, such as infections and malignancy[9]. In contrast, recent data from pooled analyses of RCTs suggest that the advanced age, and not anti-TNF exposure, was associated with increased rates of SAE and hospitalizations[10].

Furthermore, landmark trials evaluating the more recently approved biologic agents, such as vedolizumab (VDZ) or ustekinumab (UST), suggested a more beneficial overall safety profile[11,12]. However, the existing literature is limited regarding effectiveness and safety of these agents in the elderly population. A post hoc analysis of the GEMINI trials reported that in IBD patients above 55 years old the efficacy and safety of VDZ was similar to younger IBD patients, while the safety profile was not different from placebo[13]. Relatively few data are available in elderly IBD patients on the efficacy or safety on anti-TNFs and on the new biologicals. One of the first studies in elderly patients was Busquets et al[14] which performed a systematic review on efficacy and safety of anti-TNFs in the elderly, however mainly in patients with rheumatic diseases. It concluded that elderly patients on anti-TNF therapy have higher number of AEs, and similar efficacy, when compared with younger patients. The aim of our study was to measure the rates of biologic therapy sustainability in elderly IBD patients, as well as to report their effectiveness and safety profile.

MATERIALS AND METHODS

Study design

Consecutive elderly patients (aged 60 years or over) previously or currently treated with a biologic agent and followed at the McGill University Health Centre Inflammatory Bowel Diseases Center between January 2000 and January 2020 were included retrospectively. The efficacy of treatment with a biologic agent was assessed by clinical score, biochemical and endoscopy. Clinical response and remission using the Harvey-Bradshaw Index (HBI) and Mayo score were measured at baseline, 3 mo, 6-9 mo, and 12-18 mo of follow up. Patients included were patients with IBD, with an age of 60 years or older, whose current or prior treatment included biological agents (anti-TNF, VDZ or UST). Patients with contra-indications to biologic therapy, or patients with less than 3 mo follow-up were excluded. For patients with multiple biological exposure, data for the last biological therapy was collected.

Local electronic medical charts were used to identify elderly IBD patients on infliximab (IFX), adalimumab (ADAL), VDZ or UST. We collected demographic data (age, gender), comorbidities, age at diagnosis, disease duration, disease extent and phenotype (Montreal classification)[15], prior gastrointestinal surgeries, C-reactive protein, fecal calprotectin, radiological or endoscopic reports and clinical symptoms of IBD activity. Additional therapeutic variables measured were treatment duration and dosage, prior or concomitant immunosuppression, parallel steroid therapy. Comorbidity was measured by the Charlson Comorbidity Index (CCI), where a CCI of 0 represents absence of comorbidity[16]. AE, SAE, hospitalizations [duration and reasons (medical, surgical, unrelated) for hospitalization] and mortality were collected. SAE was grouped into four distinct categories: Infection (infection reported during the course of biologic therapy not requiring hospitalization), severe infection (any infection reported during the course of biologic therapy that needed hospitalization), malignancy, IBD-related surgeries (excluding elective surgical management of perianal lesions). If a treatment-related complication did not fit the previously-mentioned criteria for a SAE, then it was considered an AE, including acute infusion reactions (within one hour of dose administration), hypersensitivity reactions, non-allergic skin rash, mild infections and other AE. Patient-related data were collected through the MUHC electronic medical record (Oacis Clinical Information System).

Outcomes

The primary outcome was the drug sustainability and comparative time-dependent safety analysis in elderly patients with IBD (aged 60 years or over) on different biologic therapy. Secondary outcomes included the comparison of rates of clinical, biochemical, and endoscopic remission in elderly IBD patients according to the biologic therapy used. Clinical response was defined as a decrease in the HBI by 3 points or more from baseline for CD or a similar decrease in the partial Mayo Score (pMayo) by 3 or more for UC, while clinical remission was defined as an overall HBI of less than 5 for CD or an overall pMayo of less than 2 for UC[17].

AE or SAE occurring within three months of the last biologic dose were considered to be related to the biologic agent. SAEs were defined as potentially life-threatening or leading to death, hospitalization, or prolongation of hospitalization, or causing significant disruption in normal life functions. Infections and malignancy were separately captured. Reasons for discontinuation of the biologic agent were also evaluated.
**Statistical analysis**
Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 20.0; SPSS INC., Chicago, Illinois). Descriptive statistics were used to summarize patient and treatment-related characteristics along with means ± SD, and common themes highlighted using qualitative data analysis. Chi-square test or Fisher exact test was used to compare categorical variables while the Student’s unpaired t-test was used to compare continuous variables. Kaplan Meier curves were plotted with COX regression analysis to assess differences in drug sustainability, infections or AE stratified by the different biological agents. A $P < 0.05$ was defined as statistically significant.

**Ethical considerations**
This study was reviewed and approved by the McGill University Health Centre Research Ethics Board under the ethical approval number: 2019-5209. The research protocol conforms to ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) and local regulations. Informed consent was obtained from each patient included in the study.

**RESULTS**

**Demographic and baseline factors**
A total of 147 elderly patients with IBD were identified, including 109 patients with CD and 38 patients with UC. The majority of patients (75.5%) were diagnosed before the age of 60, thus adult-onset, elderly IBD patients. Disease location was predominantly ileocolonic (47.7%) in patients with CD and pancolitis for patients with UC (63.2%). Among patients with CD, 21.1% suffered from perianal disease. The CCI was at least 1 in 95.2% of elderly IBD patients, at least 3 in 47.6% and above 4 in 12.9%. Approximately 45.6% (67 patients) of all included patients underwent at least one surgical resection related to IBD. 70 elderly IBD patients (47.6%) had previous exposure to other biologics. Over the study period, 35.4% of patients had received a course of systemic steroids at least once, while 17% were treated with concomitant immunomodulatory therapy. The mean duration of biological therapy was 157.5 (SD = 148) wk. Extraintestinal manifestations had been diagnosed in 10.9% of elderly IBD patients. Table 1 summarizes disease characteristics and history of IBD-related therapy.

**Drug sustainability, time to AE or infection**
Figure 1A (see appendix section) shows the time to treatment discontinuation stratified by the biological agent by Kaplan-Meier and Cox regression analysis. No significant difference was found among the four biologicals ($P = 0.195$) (Figure 1A). According to Figure 1B, the time to AE was not significantly different in a Kaplan-Meier and Cox regression analysis among the four biologics ($P = 0.158$). Figure 1C shows the time to infections stratified by the biological agent. There was no statistical difference among the biologicals across the respective time to infection curves ($P = 0.973$). SAE was observed in only one patient (0.6%), who presented fever of unknown origin, needing hospitalization.

**Efficacy**
Figure 2A (see appendix section) shows the rates of clinical response and remission in elderly IBD patients treated with different biologicals according to HBI or Mayo scores at 3 mo. When assessing the clinical response in patients with CD, 71% of the patients on ADAL, 70% on UST, 65.2% on IFX and 60% on VDZ achieved clinical response at 3 mo. Regarding clinical remission in CD at 3 mo, 61.3% of patients on ADAL, followed by 54.2% on UST, 50% on VDZ and 47.8% on IFX achieved clinical remission. When looking at clinical response at 3 mo in patients with UC, 80% of the patients on ADAL, followed by VDZ with 44.4% and IFX with 40% responded. With regards to clinical remission at 3 mo, 40% patients on ADAL achieved clinical remission, 30% on IFX and 20% on VDZ.

The rates of clinical remission in elderly IBD patients treated with different biologicals according to HBI or Mayo scores at 6-9 mo are show in Figure 2B (see appendix section). Regarding clinical response in CD, 71.4% of patients on UST achieved clinical response at 6-9 mo, followed by 60.9 % on IFX, 58.3% on VDZ and 54.1 % on ADAL. As for clinical remission in CD, 56.5% of patients on IFX, 50% on UST, 45.9% on ADAL and 41.7% on VDZ achieved clinical remission at 6-9 mo. When evaluating clinical response in UC, 55.6% of patients on IFX, followed by 45.5% on VDZ, 42.9% on ADAL reached clinical response at 6-9 mo. As for clinical remission in UC, 42.9% of patients on ADAL, 36.4% on VDZ and 33.3% on IFX achieved this outcome.

The rates of clinical remission in elderly IBD patients treated with different biologicals according to HBI or Mayo scores after 12-18 mo are presented in Figure 2C (see appendix section). Regarding clinical remission in patients with CD, 55.6% of patients on ADAL, followed by 40% on VDZ, 37.5% on IFX and 30% on UST achieved remission. As for clinical remission in patients with UC, approximately 62.5% of patients on IFX, followed by 50% on ADAL and 45.5% on VDZ achieved remission. Clinical response and remission rates were not significantly different across biologicals in either CD or UC at any time points ($P = ns$ for each assessment), as shown in Figure 2 (see appendix section). Given the retrospective
### Table 1: Disease characteristics at baseline and history of inflammatory bowel disease-related therapy

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<tr>
<th>Biological therapy</th>
<th>Vedolizumab % (n = 23)</th>
<th>Adalimumab % (n = 57)</th>
<th>Infliximab % (n = 42)</th>
<th>Ustekinumab % (n = 25)</th>
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<td><strong>Concomitancy of 5-ASA</strong></td>
<td>Yes</td>
<td>39 (9)</td>
<td>14 (8)</td>
<td>8 (19)</td>
</tr>
<tr>
<td><strong>Concomitancy of systemic steroids at baseline</strong></td>
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<td>43 (10)</td>
<td>26 (15)</td>
<td>40 (17)</td>
</tr>
<tr>
<td><strong>Concomitancy of immunomodulator at baseline</strong></td>
<td>Yes</td>
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<td><strong>History of an extraintestinal manifestation</strong></td>
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The major finding of our study was that the drug sustainability and safety of the different biologicals were not significantly different in a large real-world, elderly IBD cohort treated in this single tertiary IBD center. Peyrin-Biroulet et al\cite{18} evaluated the efficacy and safety profile of anti-TNF in IBD patients, showing no difference in the frequency of mortality, malignancies and serious infections between anti-TNF and control group. Similarly, Lichtenstein et al\cite{19} reported that the occurrence of death was similar between patients treated with anti-TNF and those who received other treatments only; however, an increased risk of infections was seen in patients treated with IFX. Borren and Ananthakrishnan\cite{20} reported that older age was associated with an increased risk of malignancy compared to younger age. Elderly patients on biologics had a 3-fold increase in risk of infection compared to those who were not using biologics, yet there were no significant differences in odds of malignancy or mortality compared to older patients that were not using biologics.

**DISCUSSION**

The nature of the study and the lack of standardization of the collected measurements for endoscopic and biomarkers, the statistical analysis was inconclusive, therefore the data is not presented.
Regarding efficacy and safety profile of biological therapy in the elderly patients, Asscher et al[21] assessed the safety and effectiveness of anti-TNF therapy in IBD patients over 60 years. Elderly patients on anti-TNF therapy have an increased risk of serious infections compared with elderly IBD patients who are not on anti-TNF therapy, not compared to younger patients who receive anti-TNF, though. However, comorbidity has been shown to be an indicator of SAE in patients exposed to anti-TNF. Effectiveness was similar between elderly and younger patients. Lobatón et al[9] also evaluated efficacy and safety of anti-TNF therapy in an elderly IBD population and showed a worse short-term clinical response to anti-TNFs at 10 wk after anti-TNF initiation, meaning that the probability of drug discontinuation during the follow-up (whatever the reason) was higher; but when excluding primary nonresponse, this proportion became similar between the two groups. No differences were found in long-term efficacy among the initial responders (79.5% vs 82.8%; \( P = 0.64 \)). As for safety, a higher risk of SAE was found in elderly IBD patients treated with anti-TNFs (risk ratio = 4.7; \( P < 0.001 \)) compared to the younger patients.
younger subgroup[9]. Along with that, our study also reported statistically similar rates of 3 mo clinical response and 6-12 mo clinical response and remission among the four types of biologics studied (ADAL, IFX, VDZ and UST). Regarding safety, time to AE and to infection were also not statistically different.

The efficacy and safety of the anti-TNFs are extensively studied, less real world or comparative data are available for the new biologicals. In the landmark clinical trials, they appeared to be a safer option compared to the anti-TNFs, although in indirect comparisons. Recently, comparative efficacy and safety data became available in IBD patients. The SEAVUE study compared UST with ADAL for induction and maintenance of biological-naive patients with moderate to severe CD. With regards to safety, 34.0% of UST-treated and 40.5% of the ADAL-treated patients had infections, 2.6% and 7.2% had SAEs, and 6.3% and 11.3% had AEs leading to therapy discontinuation in non-elderly IBD patients[22]. VARSITY trial compared VDZ with ADAL in patients with moderately to severely active UC, mainly patients without previous exposure to biologics. Numerical differences were observed in the reported AEs. Of note, the exposure-adjusted incidence rate of infection was 23.4 per 100 patients-year in the VDZ group and 34.6 per 100 patients-year in the ADAL group[23].

As for the elderly IBD population on new biologicals, there is still a paucity of data concerning efficacy and safety from real world studies. Cohen et al[24] evaluated the efficacy and safety of VDZ in elderly IBD patients compared to non-elderly patients. Equal effectiveness in both groups was reported; however, there was a higher risk of infections among the elderly on VDZ, which could be related to age and due to underlying diseases[24]. Garg et al[25] evaluated the safety and efficacy of UST in elderly CD patients. Efficacy and safety were similar in this relatively small cohort in elderly and non-elderly IBD patients; elderly patients were less likely severe, though, and both groups had 95% previous exposure to biologics. Furthermore, the mucosal healing rates observed in the elderly cohort were in check with other real-world studies performed in non-elderly IBD patients. As for safety, UST use in elderly IBD was not associated with higher rates of infusion reaction, infections, or postoperative complications as compared to the non-elderly patients[25]. In line with these studies, ours showed no significant difference in time to AEs and infection among elderly IBD patients treated with anti-TNF, VDZ and UST.

The strength of our study is that represents a single center cohort with harmonized treatment and follow-up strategies across IBD specialists. In addition, a complex analysis of effectiveness and safety was performed in a relatively large elderly IBD cohort. However, the present study has limitations. First, there was a relatively low number of elderly patients on new biological therapies. Second, it consists of a retrospective cohort with intrinsic problems of accuracy and potential biases such as recall bias and reporting bias, specially of AEs and mild infections, which patients may not have announced or may not have been documented. Third, follow-up on biomarkers, fecal calprotectin and endoscopy were not uniform for timing. Fourth and last, rates of previous exposure to biologicals were different for new biologicals vs anti-TNFs.

CONCLUSION
Current biologic therapies were not different concerning drug sustainability, effectiveness, and safety in the elderly IBD population. Based on these results, a preferred sequencing order among biologicals for this specific population is not possible to be suggested thus, larger studies in elderly IBD population are warranted.

ARTICLE HIGHLIGHTS

Research background
Biologic therapy resulted in a significant positive impact on the treatment of inflammatory bowel disease (IBD) however data on the efficacy and side effects of these therapies in the elderly is scant.

Research motivation
To further evaluate and develop more studies regarding treatment efficacy and safety of biological therapies in a specific and sensitive population, such as the elderly IBD patients, since there is not much evidence about it on medical literature so far.

Research objectives
Retrospectively evaluate the drug sustainability, effectiveness, and safety of the biologic therapies in the elderly IBD population.

Research methods
Consecutive elderly (≥ 60 years old) IBD patients, treated with biologics [infliximab (IFX), adalimumab
(ADAL), vedolizumab (VDZ), ustekinumab (UST)] followed at the McGill University Inflammatory Bowel Diseases Center were included between January 2000 and 2020. Efficacy was measured by clinical scores at 3, 6-9 and 12-18 mo after initiation of the biologic therapy. Patients completing induction therapy were included. Adverse events (AEs) or serious AEs were collected during and within three months of stopping of the biologic therapy.

Research results
A total of 147 elderly patients with IBD were identified and treated with biologicals during the study period, including 109 with Crohn’s disease and 38 with ulcerative colitis. Patients received the following biologicals: IFX (28.5%), ADAL (38.7%), VDZ (15.6%), UST (17%). The mean duration of biological therapy was 157.5 (SD = 148) wk. Parallel steroid therapy was given in 34% at baseline, 19% at 3 mo, 16.3% at 6-9 mo and 6.5% at 12-18 mo. The remission rates at 3, 6-9 and 12-18 mo were not significantly different among biological therapies. Kaplan-Meyer analysis did not show statistical difference for drug sustainability ($P = 0.195$), time to adverse event ($P = 0.158$) or infection rates ($P = 0.973$) between the four studied biologicals. The most common AEs that led to drug discontinuation were loss of response, infusion/injection reaction and infection.

Research conclusions
Current biologics were not different regarding drug sustainability, effectiveness, and safety in the elderly IBD population. Therefore, it is not possible to suggest a preferred sequencing order among biologics.

Research perspectives
The authors expect that this article may help other IBD physicians and gastroenterologists in their decision process for treating elderly IBD patients with biological therapy.

FOOTNOTES

Author contributions: Hahn GD and Bessissow T are guarantors of the manuscript; LeBlanc JF and Bessissow T designed the study; Drügg Hahn G, Qatomah A, Wang A, Boodaghians L, Liu Chen Kiow J and Al Ali M performed the data collection; Drügg Hahn G, LeBlanc JF, Golovics PA, Wettwittayakhlang P, Lakatos PL and Bessissow T performed the data interpretation; Lakatos PL performed the statistical analysis; Drügg Hahn G wrote the initial draft of the manuscript, and Golovics PA, Wettwittayakhlang P, Wild G, Afif W, Bitton A, Lakatos PL and Bessissow T were involved in the critical revision of the manuscript; and all the authors reviewed and approved the final manuscript.

Institutional review board statement: The study was approved by the Research Ethics Board of McGill University Health care center, Montreal, Quebec, Canada, under protocol No. 2019-5209. Individual patient-level data were de-identified to maintain confidentiality in all steps of study analysis. This study was conducted in compliance with regulations stated in the 1975 Declaration of Helsinki.

Informed consent statement: Informed consent was obtained from each patient included in the study.

Conflict-of-interest statement: Hahn GD, Qatomah A, Wang A, Boodaghians L, Liu Chen Kiow J, Al Ali M, and Wild G declared no conflict of interest; LeBlanc JF has been a speaker or advisory board member for Janssen and Takeda; Golovics PA has been a speaker for AbbVie, Takeda, Fresenius, Ferring; Wettwittayakhlang P has been a speaker and/or advisory board member: Takeda, Pfizer, Janssen, Ferring, A. Menerini, and MSD; Afif W has been a speaker for Janssen, Prometheus, Dynacare, Takeda, AbbVie Theradiag; Bitton A has been a member of Advisory Boards - Abbvie, Pfizer, Takeda, Janssen, Merck; Speaker’s bureau - Abbvie; Janssen, Takeda, Pfizer; Lacatos PL has been a speaker and/or advisory board member for AbbVie, Arena, Falk Pharma GmbH, Ferring, Genetech, Janssen, Kyowa Hakko Kirin Pharma, Mitsubishi Tanabe Pharma Corporation, MSD, Pfizer, Roche, Shire, Takeda and Tillots, and has received unrestricted research grants from AbbVie, MSD and Pfizer; Bessissow T has been a speaker or advisory board member for Takeda, Janssen, Abbvie, Merck, Pfizer, Pendopharm, Ferring, Shire, Sandoz, BMS, Roche, Fresenius Kabi, Viatris.

Data sharing statement: The main data are given in this article. The data are available from the corresponding author upon request.

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

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Country/Territory of origin: Canada

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

REFERENCES


Prevalence and factors associated with vitamin C deficiency in inflammatory bowel disease

Benjamin Langan Gordon, Jonathan S Galati, Stevie Yang, Randy S Longman, Dana Lukin, Ellen J Scherl, Robert Battat

BACKGROUND
Patients with inflammatory bowel disease (IBD) are prone to several nutritional deficiencies. However, data are lacking on vitamin C deficiency in Crohn’s disease (CD) and ulcerative colitis (UC) patients, as well as the impact of clinical, biomarker and endoscopic disease severity on the development of vitamin C deficiency.

AIM
To determine proportions and factors associated with vitamin C deficiency in CD and UC patients.

METHODS
In this retrospective study, we obtained clinical, laboratory and endoscopic data from CD and UC patients presenting to the IBD clinic at a single tertiary care center from 2014 to 2019. All patients had an available plasma vitamin C level. Of 353 subjects who met initial search criteria using a cohort discovery tool, 301 ultimately met criteria for inclusion in the study. The primary aim described vitamin C deficiency (≤ 11.4 μmol/L) rates in IBD. Secondary analyses compared proportions with deficiency between active and inactive IBD. Multivariate logistic regression analysis evaluated factors associated with deficiency.

RESULTS
Of 301 IBD patients, 21.6% had deficiency, including 24.4% of CD patients and 16.0% of UC patients. Patients with elevated C-reactive protein (CRP) (39.1% vs 16.9%, P < 0.001) and fecal calprotectin (50.0% vs 20.0%, P = 0.009) had signifi-
cantly higher proportions of deficiency compared to those without. Penetrating disease \( (P = 0.03) \), obesity \( (P = 0.02) \) and current biologic use \( (P = 0.006) \) were also associated with deficiency on univariate analysis. On multivariate analysis, the objective inflammatory marker utilized for analysis (elevated CRP) was the only factor associated with deficiency (odds ratio = 3.1, 95% confidence interval: 1.5-6.6, \( P = 0.003 \)). There was no difference in the presence of clinical symptoms of scurvy in those with vitamin C deficiency and those without.

**CONCLUSION**

Vitamin C deficiency was common in IBD. Patients with elevated inflammatory markers and penetrating disease had higher rates of vitamin C deficiency.

**Key Words:** Inflammatory bowel disease; Crohn’s disease; Ulcerative colitis; Vitamin C deficiency; Scurvy; Malnutrition

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**Core Tip:** This study aimed to determine proportions and factors associated with vitamin C deficiency in inflammatory bowel disease (IBD) patients. In 301 patients, 21.6% had vitamin C deficiency, including 24.4% of Crohn’s disease and 16.0% of ulcerative colitis patients. Patients with elevated C-reactive protein (39.1% vs 16.9%) and fecal calprotectin (50.0% vs 20.0%) had higher proportions of deficiency compared to those without, as did patients with penetrating disease (36.2% vs 20.8%). This study provides the largest data on vitamin C deficiency in IBD, and demonstrates that deficiency is common in this population, particularly those with markers of active luminal or penetrating disease.

**Citation:** Gordon BL, Galati JS, Yang S, Longman RS, Lukin D, Scherl EJ, Battat R. Prevalence and factors associated with vitamin C deficiency in inflammatory bowel disease. *World J Gastroenterol* 2022; 28(33): 4834-4845

**URL:** https://www.wjgnet.com/1007-9327/full/v28/i33/4834.htm

**DOI:** https://dx.doi.org/10.3748/wjg.v28.i33.4834

**INTRODUCTION**

Inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract that affect over 1.5 million people in the United States alone[1,2]. Several nutritional deficiencies are well described in patients with IBD, the most common being iron, vitamin B12, vitamin D, zinc, and calcium[3-7]. However, far less literature exists on vitamin C (ascorbic acid) deficiency in this population. While the prevalence of scurvy - the clinical manifestations of vitamin C deficiency - has largely declined in the 21st century, up to 7% of the United States population still possesses vitamin C deficiency. The risk of deficiency is particularly increased in smokers, obese patients, and patients from low-income backgrounds[8-11]. Among those also at risk are patients with poor vitamin C intake and malabsorptive processes.

Traditionally, in IBD patients, vitamin C deficiency is thought to originate from insufficient consumption, malabsorption, and altered metabolism of vitamin C. Although not routinely recommended, many IBD patients adhere to low-residue diets to decrease gut motility and bacterial fermentation of fiber. Unfortunately, several regimens exclude fresh fruit and vegetables, the main dietary sources of vitamin C[9]. Inadequate consumption is often compounded by malabsorption of vitamin C in these patients. Ascorbate is primarily absorbed in the jejunum and ileum, which are often affected in CD[10]. In addition, patients with IBD have been shown to have polymorphisms in genes encoding vitamin C transporters necessary for vitamin C uptake[11,12]. Lastly, tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine often elevated in IBD, also downregulates transcription of transporters necessary for vitamin C uptake[13,14].

Vitamin C deficiency can lead to impaired uptake and utilization of iron, poor wound healing, and bleeding[15,16]. The diagnosis of vitamin C deficiency can be all the more challenging to make in patients with IBD, as many nonspecific symptoms of scurvy - fatigue, arthralgias, and cutaneous manifestations - can confound systemic symptoms of CD and UC. Prior studies of patients with IBD have described inadequate vitamin C intake and suboptimal serum vitamin C levels in 22%-70% and 15%-84%, respectively[9,17-20]. Notably, these studies have been small, excluded patients with UC, and occurred prior to the advent of biologic medications. Importantly, cohorts lacked data on clinical, biomarker and endoscopic measures of disease activity to assess for their impact on vitamin C deficiency.
IBD patients are at risk for malnutrition and vitamin C deficiency is an easily reversible condition. Thus, it is essential to understand the prevalence of and factors associated with vitamin C deficiency in this population to better identify those at risk. For this reason, this study aimed to determine rates of vitamin C deficiency in patients with CD and UC and investigate potential factors associated with the development of vitamin C deficiency in this population.

**MATERIALS AND METHODS**

**Patient population**

Data were extracted from chart review of patients presenting to the IBD clinic at a single tertiary institution from 2014 to 2019. Patients were identified using a cohort discovery tool (Informatics for Integrating Biology and the Bedside, National Center for Biomedical Computing, Partners HealthCare System, Boston, Massachusetts) at Weill Cornell Medicine, New York. Search criteria included age 18 years and older, diagnosis of CD or UC, and a plasma vitamin C measurement drawn from 2014 to 2019. International Classification of Diseases 10th edition codes were used to identify patients with CD (K50.x) and UC (K51.x). Exclusion criteria included lack of a CD or UC diagnosis, plasma vitamin C level, or IBD-related visit at the time that plasma vitamin C measurement was performed. Three hundred fifty-three subjects matched initial search criteria. In patients with multiple plasma vitamin C levels, the lowest value and associated visit were utilized. This study was conducted retrospectively from data obtained for clinical purposes. The study was approved by the institutional review board at Weill Cornell Medicine, who confirmed that no ethical approval was required.

**Data extraction**

We extracted covariates readily available in the electronic medical record. Baseline characteristics were collected, including age, sex, race, body mass index (BMI), smoking history, type of IBD (CD or UC), disease duration and prior IBD-related surgeries (i.e., total proctocolectomy, ileocolonic resection, small bowel resection, etc.). Endoscopic scores - within six months of plasma vitamin C level assessment - were collected when available. Disease location and behavior were defined by the Montreal classification for IBD [21]. Patients were evaluated for current and prior IBD medications, including biologic agents such as TNF-α inhibitors (infliximab, adalimumab, golimumab, and certolizumab pegol), vedolizumab, and ustekinumab. Additional laboratory values collected within one week of plasma vitamin C levels were included in the analysis. When available, C-reactive protein (CRP), iron, transferrin saturation, ferritin, vitamin B12, vitamin D, 25-hydroxy, and fecal calprotectin were obtained. Iron deficiency was defined as ferritin < 30 ng/mL and transferrin saturation < 16% in those with quiescent disease and ferritin < 100 ng/mL and transferrin saturation < 16% in those with active disease [22]. Vitamin B12 deficiency was defined as serum vitamin B12 level < 200 pg/mL or < 148 pmol/L [7]. Vitamin D deficiency or insufficiency was defined as serum vitamin D, 25-hydroxy level ≤ 20 ng/mL [23]. From the electronic medical record, we also extracted data on symptoms typically associated with vitamin C deficiency (i.e., scurvy) at the IBD visit when plasma vitamin C level was obtained. These included fatigue, arthritis/arthralgias, skin findings (rash, hyperpigmentation, etc.), easy bruising, gingivitis, poor wound healing, perifollicular findings (hemorrhage, folliculitis) and alopecia.

**Outcomes and definitions**

The primary study outcome was the prevalence of vitamin C deficiency in IBD patients. Vitamin C deficiency was defined as plasma vitamin C level < 11.4 μmol/L [24,25]. Inadequate vitamin C level or marginal deficiency was defined as 11.4-28.0 μmol/L, consistent with prior studies [24,25]. Secondary analyses were performed to assess whether clinical, biomarker or endoscopic disease activity were associated with deficiency. Patients were assessed for clinical disease activity using the Harvey-Bradshaw Index for CD [26] and modified partial Mayo score for UC [27]. Clinically active disease was defined as Harvey-Bradshaw Index > 5 for CD. For UC, clinically active disease was defined as either stool frequency or rectal bleeding > 1 on the modified partial Mayo score. Elevated CRP was defined as > 0.9 mg/dL and elevated fecal calprotectin was defined as > 250 μg/g. Endoscopically severe disease was defined as simple endoscopic score CD > 15 for CD and Mayo endoscopic score ≥ 2 for UC [28-30]. Additional outcomes based on biologic plausibility included the association of vitamin C deficiency with IBD type (CD or UC), obesity (BMI ≥ 30), use of biologic medications, disease location in the small intestine, penetrating disease, IBD-related surgery, elevated CRP, elevated fecal calprotectin, iron deficiency, clinically active disease and endoscopically severe disease.

**Statistical analysis**

The primary outcome described the prevalence of vitamin C deficiency in patients with IBD. To address this outcome, based on previous data in 137 CD patients showing a 15% prevalence of vitamin C deficiency [17], in our exploratory cohort of 301 patients, we expected that a two-sided 95% confidence interval (CI) for the prevalence could be constructed to be within ± 4.0% of the observed prevalence.
For secondary outcomes, variables (listed above) were selected based on biologic plausibility for abnormal vitamin C absorption. To address these secondary outcomes, we performed chi-squared tests or Fisher’s exact tests as appropriate to compare the proportions of vitamin C deficiency between groups. A multivariate logistic regression was performed on covariates selected based on biologic plausibility. These included presence of small bowel disease, penetrating disease, history of IBD-related surgery, obesity, current biologic medication use, elevated CRP, and clinically active disease. Variables with $P \leq 0.2$ were selected for inclusion in the final model. CRP was selected as the sole objective inflammatory marker for sample size considerations (i.e., fecal calprotectin and endoscopy data had limited sample size) and to avoid multicollinearity with these other inflammatory assessments. All analyses were performed using Stata Version 16.0 (StataCorp, College Station, TX). Continuous variables were expressed as means ± SD. The multivariate analysis was expressed as odds ratio (OR) with 95% CI.

### RESULTS

#### Baseline demographics

A total of 301 CD or UC patients with available plasma vitamin C levels were included in the study. Baseline characteristics of the entire cohort are described in Table 1. The mean age of the cohort was 47.6 ± 17.4 years. One hundred ninety (63.1%) subjects were female, and 230 (76.4%) were Caucasian. A total of 201 (66.8%) patients had a diagnosis of CD and 100 (33.2%) had a diagnosis of UC. The mean duration of disease was 17.0 ± 13.6 years. A total of 109 (36.2%) patients had a history of IBD-related surgery and 133 patients (44.2%) were undergoing treatment with a biologic agent at the time of plasma vitamin C level collection. Six patients (2.0%) were active smokers and 42/291 (14.4%) had a BMI ≥ 30. Fifty-nine of 201 (66.8%) patients had clinical active disease. Of 109 patients with available endoscopy, 20 (17.7%) had endoscopically severe disease.

#### Proportion of IBD patients with vitamin C deficiency

The mean vitamin C level was 35.7 ± 27.8 μmol/L in the entire IBD cohort. For analysis of the primary outcome, 21.6% of IBD patients (65/301) had vitamin C deficiency (< 11.4 μmol/L). An additional 24.6% of IBD patients (74/301) had inadequate vitamin C levels (11.4-28.0 μmol/L). CD patients had numerically higher prevalence of vitamin C deficiency than those with UC, although this result did not reach statistical significance (24.4% vs 16.0%, $P = 0.1$, Figure 1).

#### Secondary outcomes: Factors associated with vitamin C deficiency in all IBD patients

In all IBD patients, those with elevated CRP had higher proportions of vitamin C deficiency (39.1% vs 16.9%, $P < 0.001$, Table 2) compared to those without elevated CRP. Similarly, patients with elevated fecal calprotectin had higher rates of vitamin C deficiency (50.0% vs 20.0%, $P = 0.009$) compared to those without fecal calprotectin elevation. In a subgroup with available endoscopic data, those with severe inflammation (n = 20) had numerically higher deficiency rates compared to those without severe inflammation (35.0% vs 22.6%, $P = 0.2$). However, comparable rates of deficiency existed between those with and without clinically active disease (26.1% vs 18.4%, $P = 0.1$). Obesity (35.7% vs 19.7%, $P = 0.02$) and current biologic medication use (28.6% vs 15.6%, $P = 0.006$) were associated with increased rates of vitamin C deficiency on univariate analysis. Among patients on current biologic therapy (n = 133), there were higher proportions of vitamin C deficiency in those using TNF-α inhibitors (17/48) compared with those using non-TNF-α biologics (35.4% vs 18.8%, $P = 0.03$). Iron deficiency (28.8% vs 20.2%, $P = 0.2$), vitamin D deficiency/insufficiency (29.9% vs 21.3%, $P = 0.3$), surgery (25.7% vs 19.3%, $P = 0.2$), and active smoking (50.0% vs 21.0%, $P = 0.1$) were not associated with higher deficiency rates. On multivariate analysis, elevated CRP was the only factor significantly associated with vitamin C deficiency (OR = 3.1, 95% CI: 1.5-6.6, $P = 0.003$). Presence of small bowel disease, penetrating disease, history of IBD-related surgery, obesity, use of a biologic agent, and clinically active disease were not.

#### Secondary outcomes: Factors associated with vitamin C deficiency stratified by disease

Among CD patients, patients with penetrating disease had significantly higher rates of vitamin C deficiency compared to patients without penetrating disease (36.2% vs 20.8%, $P = 0.03$, Table 2). In CD patients, both elevated CRP (41.2% vs 17.9%, $P = 0.001$) and fecal calprotectin (56.3% vs 26.3%, $P = 0.04$) were persistently associated with higher proportions of vitamin C deficiency compared to those without elevated biomarkers. In subgroups of CD patients with endoscopic data available, those with endoscopically severe disease (n = 12) had numerically increased prevalence of vitamin C deficiency (41.7% vs 27.7%, $P = 0.3$). Similarly, CD patients with clinically active disease had numerically higher rates of deficiency (30.2% vs 20.2%, $P = 0.1$). CD patients with small bowel involvement did not have higher
Table 1 Baseline characteristics

<table>
<thead>
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<tr>
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</tr>
<tr>
<td>Left-sided colitis</td>
<td>45 (47.4%)</td>
</tr>
<tr>
<td>Pancolitis</td>
<td>36 (37.9%)</td>
</tr>
<tr>
<td>Disease duration (yr)</td>
<td>17.0 ± 13.6</td>
</tr>
<tr>
<td>IBD related surgery</td>
<td>109 (36.2%)</td>
</tr>
<tr>
<td>IBD medications</td>
<td></td>
</tr>
<tr>
<td>Current biologic use</td>
<td>133 (44.2%)</td>
</tr>
<tr>
<td>Past/ever biologic use</td>
<td>166 (55.1%)</td>
</tr>
<tr>
<td>Clinically active disease¹</td>
<td>134 (45.9%)</td>
</tr>
<tr>
<td>CD</td>
<td>96 (49.2%)</td>
</tr>
<tr>
<td>UC</td>
<td>38 (59.2%)</td>
</tr>
<tr>
<td>Endoscopically severe disease¹</td>
<td>20 (17.7%)</td>
</tr>
<tr>
<td>CD</td>
<td>12 (15.6%)</td>
</tr>
<tr>
<td>UC</td>
<td>8 (22.2%)</td>
</tr>
</tbody>
</table>

¹Ten patients had unknown body mass index. Five ulcerative colitis (UC) patients had unknown disease location. Nine patients did not have disease activity available (6 Crohn’s disease, 3 UC). One hundred thirteen inflammatory bowel disease patients had endoscopies available. Clinically active disease was defined as Harvey-Bradshaw index > 5 for Crohn’s disease (CD), and stool frequency or rectal bleeding > 1 on modified partial Mayo score for ulcerative colitis (UC). Endoscopically severe disease was defined as simple endoscopic score CD > 15 in CD and endoscopic Mayo
score ≥ 2 in UC. IBD: Inflammatory bowel disease; CD: Crohn’s disease; UC: Ulcerative colitis; BMI: Body mass index.

### Table 2 Prevalence of vitamin C deficiency in covariate populations

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Prevalence of vitamin C deficiency in patients with covariate</th>
<th>Prevalence of vitamin C deficiency in patients without covariate</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>49/201 (24.4%)</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>UC</td>
<td>16/100 (16.0%)</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Small bowel disease</td>
<td>38/165 (23.0%)</td>
<td>11/36 (30.6%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Penetrating disease</td>
<td>17/47 (36.2%)</td>
<td>32/154 (20.8%)</td>
<td>0.03</td>
</tr>
<tr>
<td>IBD related surgery</td>
<td>28/109 (25.7%)</td>
<td>37/192 (19.3%)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD</td>
<td>25/96 (26.0%)</td>
<td>24/105 (22.9%)</td>
<td>0.6</td>
</tr>
<tr>
<td>UC</td>
<td>3/13 (23.1%)</td>
<td>13/87 (14.9%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Obesity (BMI ≥ 30)</td>
<td>15/42 (35.7%)</td>
<td>49/249 (19.7%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Active smoking</td>
<td>3/6 (50.0%)</td>
<td>62/295 (21.0%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Current biologic use</td>
<td>38/133 (28.6%)</td>
<td>26/167 (15.6%)</td>
<td>0.006</td>
</tr>
<tr>
<td>CRP &gt; 0.9 mg/dL</td>
<td>25/64 (39.1%)</td>
<td>36/213 (16.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD</td>
<td>21/51 (41.2%)</td>
<td>24/134 (17.9%)</td>
<td>0.001</td>
</tr>
<tr>
<td>UC</td>
<td>4/13 (30.8%)</td>
<td>12/79 (15.2%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Fecal calprotectin &gt; 250 ug/g</td>
<td>10/20 (50.0%)</td>
<td>12/60 (20.0%)</td>
<td>0.009</td>
</tr>
<tr>
<td>CD</td>
<td>9/16 (56.3%)</td>
<td>10/38 (26.3%)</td>
<td>0.04</td>
</tr>
<tr>
<td>UC</td>
<td>1/4 (25.0%)</td>
<td>2/22 (9.1%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Iron deficiency</td>
<td>17/59 (28.8%)</td>
<td>39/193 (20.2%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin D deficiency/insufficiency</td>
<td>11/38 (28.9%)</td>
<td>47/221 (21.3%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Clinically active disease</td>
<td>35/134 (26.1%)</td>
<td>29/158 (18.4%)</td>
<td>0.1</td>
</tr>
<tr>
<td>CD</td>
<td>29/96 (30.2%)</td>
<td>20/99 (20.2%)</td>
<td>0.1</td>
</tr>
<tr>
<td>UC</td>
<td>6/38 (15.8%)</td>
<td>9/59 (15.3%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Endoscopically severe disease</td>
<td>7/20 (35.0%)</td>
<td>21/93 (22.6%)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD</td>
<td>5/12 (41.7%)</td>
<td>18/65 (27.7%)</td>
<td>0.3</td>
</tr>
<tr>
<td>UC</td>
<td>2/8 (25.0%)</td>
<td>3/28 (10.7%)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1Patients with penetrating disease, obesity, biologic use, elevated C-reactive protein and elevated fecal calprotectin had increased frequency of vitamin C deficiency. Clinically active disease was defined as Harvey-Bradshaw index > 5 for Crohn’s disease (CD), and stool frequency or rectal bleeding > 1 on modified partial Mayo score for ulcerative colitis (UC). Endoscopically severe disease was defined as simple endoscopic score CD > 15 in CD and endoscopic Mayo score ≥ 2 in UC. IBD: Inflammatory bowel disease; CD: Crohn’s disease; UC: Ulcerative colitis; BMI: Body mass index; CRP: C-reactive protein.

Rates of vitamin C deficiency compared to those without small bowel involvement (23.0% vs 30.6%, P = 0.3).

In small subgroups of UC patients with available data, numerical differences persisted between those with elevated CRP (n = 13) and those with elevated fecal calprotectin (n = 4) when compared to those without elevated biomarkers (CRP: 30.8% vs 15.2%, P = 0.2; fecal calprotectin: 25.0% vs 9.1%, P = 0.4). In a small subset of UC patients with available endoscopic data, those with endoscopically severe disease (n = 8) had numerically increased frequency of vitamin C deficiency (25.0% vs 10.7%, P = 0.3). However, clinically active disease was not associated with higher rates of vitamin C deficiency when compared to those with quiescent disease (15.8% vs 15.3%, P = 0.9).
Symptoms of vitamin C deficiency
When comparing patients with and without vitamin C deficiency, there was no difference in the presence of one or more clinical features of scurvy (66.2% vs 58.5%, $P = 0.3$, Table 3). Furthermore, both groups had similar rates of arthritis, cutaneous findings, easy bruising, gingivitis, perifollicular findings and alopecia. Patients with vitamin C deficiency were more likely to report fatigue than those with normal vitamin C levels (43.1% vs 27.5%, $P = 0.02$). Moreover, vitamin C deficient patients were more likely to report poor wound healing (4.6% vs 0.4%, $P = 0.03$).

DISCUSSION
Though many consider scurvy a historical disease of seafarers, the current study demonstrates that vitamin C deficiency affects a significant minority of IBD patients. In 301 patients, 21.6% of IBD patients had vitamin C deficiency, including 24.4% of CD patients and 16.0% of UC patients. This is approximately three-fold higher than the prevalence of vitamin C deficiency in the overall United States population[8].

Strikingly, in IBD patients with elevated objective markers of inflammation, such as CRP and fecal calprotectin, vitamin C deficiency rates ranged from 39%-50%. Similarly, CD patients with penetrating phenotype had higher deficiency rates. On multivariate analysis, the association between elevated CRP and vitamin C deficiency persisted. To our knowledge, this study uniquely examines the relationship between objectively quantified intestinal inflammation (using endoscopy, $n = 113$, or fecal calprotectin, $n = 80$) and vitamin C deficiency in a large cohort. Subgroup analysis in patients with available endoscopic data was concordant with biomarker data, with numerically higher rates of deficiency in those with significant intestinal inflammation. In UC, biomarkers and endoscopic data were more limited, with few patients in groups with elevated CRP, fecal calprotectin and endoscopic inflammation available. Absolute rates of deficiency were non-significantly lower in UC, but numerical differences between UC patients with and without inflammation were similar to these differences in CD. Notably, even in patients without objective evidence of inflammatory disease - based on CRP, calprotectin, and endoscopic score - rates of deficiency ranged from 17%-23%.

This study is the largest to date to report on the prevalence of vitamin C deficiency in IBD. Additionally, the current study significantly increases available data in both CD and UC by utilizing a well characterized cohort to provide analyses on factors associated with deficiency. Previous smaller studies have shown vitamin C deficiency rates of 15%-84% in CD patients[9,17-20]. The variability of decreased vitamin C levels in these studies largely stems from differences in sample sizes and reference ranges.

While previous studies report inadequate vitamin C intake in UC[31], to our knowledge, there are no prior studies describing proportions with vitamin C deficiency in UC. Vitamin C deficiency would be biologically plausible in CD as CD often affects the primary sites of vitamin C absorption in the small bowel. Interestingly, in UC patients (without small bowel disease), 16% had vitamin C deficiency. While dietary data was not available in this study, avoidance of vitamin C rich foods likely contributed to the development of vitamin C deficiency in patients with UC, as has been reported in previous studies[31]. Moreover, patients with UC often have elevated TNF-$\alpha$, which has been shown to downregulate...
There was no difference in presence of clinical features of scurvy between patients with vitamin C deficiency and those without. Patients with vitamin C deficiency were more likely to report fatigue and poor wound healing.

Table 3 Clinical features of vitamin C deficiency

<table>
<thead>
<tr>
<th>Presence of ≥ 1 clinical feature(s) of scurvy</th>
<th>Vitamin C deficiency (n = 65)</th>
<th>Normal vitamin C level (n = 236)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>22 (33.8%)</td>
<td>98 (41.5%)</td>
<td>-</td>
</tr>
<tr>
<td>Fatigue</td>
<td>28 (43.1%)</td>
<td>65 (27.5%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Arthritis/arthralgias</td>
<td>27 (41.5%)</td>
<td>96 (40.7%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Skin findings (rash, hyperpigmentation)</td>
<td>9 (13.8%)</td>
<td>29 (12.3%)</td>
<td>0.7</td>
</tr>
<tr>
<td>Easy bruising</td>
<td>6 (9.2%)</td>
<td>9 (3.8%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>5 (4.6%)</td>
<td>6 (2.5%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Poor wound healing</td>
<td>3 (4.6%)</td>
<td>1 (0.4%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Perifollicular findings (hemorrhage, folliculitis)</td>
<td>2 (3.1%)</td>
<td>1 (0.4%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Alopecia</td>
<td>1 (1.5%)</td>
<td>5 (2.1%)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1Patients with vitamin C deficiency were more likely to report fatigue and poor wound healing. There was no difference in presence of clinical features of scurvy between patients with vitamin C deficiency and those without.

transporters involved in vitamin C uptake[13,14]. The current study found penetrating disease to be associated with vitamin C deficiency. Previously, development of metabolic bone disease has been associated with a penetrating phenotype[32], although few studies have commented on the development of micronutrient deficiency in CD patients with penetrating disease. Penetrating disease often involves the small bowel and also likely reflects more active, refractory CD. Thus, patients with penetrating phenotypes may be at higher risk of both malabsorption - via inflamed tissue and enteric fistulas - and poor consumption - via dietary avoidance of foods rich in vitamin C that may exacerbate symptoms. These data are consistent with CRP and fecal calprotectin elevations also being associated with deficiency. Interestingly, presence of small bowel disease was not associated with increased risk of vitamin C deficiency in CD, despite the jejunum and ileum being the primary sites of vitamin C absorption. However, historical disease location may have been confounded by patients with inactive small bowel disease. Further studies on patients with active small intestinal disease would be required prior to concluding a lack of association between disease location and deficiency status.

While obesity and biologic medication use have biologic plausibility for deficiency and were associated on univariate analysis[8,13,14,33], the study results in aggregate more strongly support active IBD being associated with vitamin C deficiency. Only CRP was associated with vitamin C deficiency on multivariate analysis. However, it should be noted that in non-IBD populations[8], obesity has been shown to be associated with higher rates of vitamin C deficiency. Prior studies suggest that increased access to low-cost, high-calorie, micronutrient-poor food may explain the association between obesity and multiple vitamin deficiencies[33]. The association of current biologic medication use and vitamin C deficiency is less clear and may be due to this being a marker of a more severe disease course. In a subgroup analysis of patients using biologic therapy, patients on TNF-α inhibitors had higher rates of deficiency compared to those on non-TNF-α agents (i.e., vedolizumab, ustekinumab, etc.), which runs counter to our understanding of TNF-α in vitamin C deficiency. TNF-α is known to downregulate transcription of transporters required for vitamin C uptake[13,14], and thus, one might expect that patients using TNF-α inhibitors would have lower, not higher proportions of deficiency. This further supports the use of anti-TNF agents or biologics as a surrogate for disease severity. Future studies may be warranted to better investigate this mechanism.

This study also highlights the difficulty in making a diagnosis of vitamin C deficiency in patients with IBD. In our cohort, there was no difference in the presence of clinical features of scurvy in patients with vitamin C deficiency compared to those with normal vitamin C levels. Many sequelae of vitamin C deficiency are nonspecific and can mimic or coexist with active IBD, including fatigue, arthralgias, oral lesions, bleeding, poor wound healing, anemia, and iron deficiency[15]. Unfortunately, more specific findings in scurvy, such as perifollicular hemorrhage and follicular hyperkeratosis, occur in only a small minority of vitamin C deficient patients, as our study reiterates. Given the challenge of diagnosing scurvy in this population, providers should have a low threshold to test for vitamin C deficiency and counsel on adequate vitamin C intake. Unlike the relapsing and often refractory nature of IBD in many patients, vitamin C supplementation can lead to rapid resolution of symptoms, including some incorrectly ascribed to IBD. Even in IBD patients with unmeasured vitamin C levels, empiric supplementation is not unreasonable, given vitamin C’s role as an antioxidant, preventing free radical damage and reducing extracellular oxidants[24]. However, future studies demonstrating that vitamin C supple-
mentation can decrease inflammatory burden or improve clinical symptoms would be necessary prior to recommending empiric supplementation as standard of care for this population.

Study limitations include the use of retrospective chart review. This study did not find an association between clinical disease severity and vitamin C deficiency. However, clinical disease indices, particularly in CD, poorly correlate with mucosal disease[34]. Though this study examined the relationship between endoscopic activity and vitamin C deficiency, analyses on this relationship were limited by the small number of patients with significant endoscopic inflammation (n = 20). Yet, numerical differences based on endoscopic inflammation were consistent with CRP and fecal calprotectin data, suggesting that intestinal inflammation impacts vitamin C deficiency. Multivariate analyses utilized a single objective marker of inflammation to avoid multicollinearity. CRP was selected as few patients had elevated fecal calprotectin (n = 20) or significant endoscopic inflammation (n = 20), whereas 277 patients had CRP data available. Additionally, given the retrospective nature of our study, data are restricted to patients who had plasma vitamin C measurements available; these patients were not necessarily being screened for deficiency. Selection bias may exist as such laboratory values may have restricted the population to those more prone to have vitamin C deficiency. Nonetheless, nearly 40% of our study population had no symptoms of scurvy when their vitamin C level was obtained, indicating a sizable component of our cohort were simply being monitored for standard nutritional deficiencies. An additional limitation of this study was that measurements of all micronutrients were not performed. The retrospective nature of our study also limits our examination of whether inadequate consumption was associated with higher rates of deficiency, or whether fasting status at serum collection impacted vitamin C level, as dietary data was not available. The use of chart review to assess for symptoms of vitamin C deficiency is also a limitation that may have led to under-detection of symptoms related to deficiency. Some providers may not routinely screen for symptoms related to scurvy (i.e., gingivitis, alopecia, etc.) and these items may not be reflected in providers' notes. Thus, reporting bias may exist. Despite this, vitamin C deficiency symptoms were infrequently documented. Lastly, this cohort was comprised of patients at an IBD center affiliated with a tertiary care center. Thus, the subjects in this study may have more severe disease, potentially impacting the generalizability of these data.

CONCLUSION

The current study demonstrates that vitamin C deficiency exists in a significant portion of patients with IBD, particularly those with objective markers of active luminal or penetrating disease. Clinical features of scurvy did not differ between patients with and without deficiency, reinforcing the challenge of diagnosing scurvy in this population, as symptoms of vitamin C deficiency and IBD may overlap. In summary, vitamin C deficiency exists in a considerable fraction of IBD patients. Thus, identifying and treating this easily reversible condition in these patients is essential.

ARTICLE HIGHLIGHTS

Research background
Patients with inflammatory bowel disease (IBD) are prone to several nutritional deficiencies, including iron, vitamin B12 and vitamin D. However, there is a lack of data on vitamin C deficiency in this population, as well as the impact of clinical, biomarker and endoscopic disease severity on the development of vitamin C deficiency.

Research motivation
As IBD patients are already at risk of malnutrition and as vitamin C deficiency is an easily reversible condition, it would be valuable to understand the prevalence of and factors associated with vitamin C deficiency in this population.

Research objectives
The primary objective assessed the prevalence of vitamin C deficiency in IBD patients. Secondary objectives evaluated proportions with deficiency between active and inactive IBD - using clinical, laboratory and endoscopic data - to better identify those at risk of deficiency.

Research methods
In this retrospective study, clinical, laboratory and endoscopic data were collected from all Crohn’s disease (CD) and ulcerative colitis (UC) patients who had available plasma vitamin C levels presenting to the IBD clinic at a single tertiary care center from 2014 to 2019. Of 353 subjects who met initial search criteria using a cohort discovery tool, 301 ultimately met criteria for inclusion in the study. The primary aim described vitamin C deficiency (≤ 11.4 μmol/L) rates in IBD, with secondary analyses comparing
proportions with deficiency between active and inactive IBD. Multivariate logistic regression analysis evaluated factors associated with deficiency.

**Research results**
In 301 IBD patients, 21.6% had vitamin C deficiency, including 24.4% of CD and 16.0% of UC patients. Patients with elevated C-reactive protein (CRP) (39.1% vs 16.9%, \( P < 0.001 \)) and fecal calprotectin (50.0% vs 20.0%, \( P = 0.009 \)) had higher proportions of deficiency compared to those without. Other factors associated with vitamin C deficiency included the presence of penetrating disease (\( P = 0.03 \)), obesity (\( P = 0.02 \)) and current biologic medication use (\( P = 0.006 \)). On multivariable analysis, the objective inflammatory marker utilized for analysis (CRP) was the only factor associated with deficiency (odds ratio = 3.1, 95% confidence interval: 1.5-6.6, \( P = 0.003 \)).

**Research conclusions**
This study provides the largest data on vitamin C deficiency in patients with IBD, uniquely assesses factors associated with deficiency and provides rigorous assessment of inflammatory status using objective markers. Vitamin C deficiency was common in IBD, particularly those with objective markers of active luminal or penetrating disease. As vitamin C deficiency exists in over one-fifth of IBD patients, it is essential to identify and treat this easily reversible condition in this population.

**Research perspectives**
Future prospective studies with well characterized cohorts, and data on diet, other micronutrient deficiencies, endoscopic assessment, and vitamin C supplementation, may be warranted to further elucidate factors associated with vitamin C deficiency and the impact of supplementation on clinical course in IBD patients.

**FOOTNOTES**

**Author contributions:** Battat R is the guarantor of the article; Gordon BL, Galati JS, Longman RS, Lukin D, Scherl EJ and Battat R contributed to the design of the study; Gordon BL, Galati JS, and Yang S collected the data; Gordon BL and Battat R analyzed the data; Gordon BL, Scherl EJ, Lukin D and Battat R wrote the paper; and all authors read and approved the final version of the manuscript.

**Institutional review board statement:** This study was reviewed and approved by the institutional review board at Weill Cornell Medicine.

**Informed consent statement:** This study was conducted retrospectively from data obtained for clinical purposes. The study was approved by the institutional review board at Weill Cornell Medicine, who confirmed that no ethical approval or informed consent was required.

**Conflict-of-interest statement:** Gordon BL, Galati JS, Yang S have none to report; Longman RS consulted Pfizer, Bristol Myers Squibb; Lukin D consults for Boehringer Ingelheim, Palatin Technologies, Pfizer; research support: AbbVie, Janssen, Kenneth Rainin Foundation, Takeda; Scherl EJ consulted AbbVie, Crohn’s and Colitis Foundation of America (CCFA), Entera Health, Evidera, GI Health Foundation, Janssen, Protagonist, Seres, Takeda, Bristol Myers Squibb; research support: AbbVie, AstraZeneca, CCFA, Janssen, Pfizer, National Institute of Health, New York Crohn’s Foundation, UCSF-CCFA Clinical Research Alliance, Genentech, Seres, Celgene, UCB, Johns Hopkins University, National Institute of Diabetes and Digestive and Kidney; Shareholder: Gilead; Honoraria: GI Health Foundation, Janssen; Battat R provide research support, fund for the Future Award and Jill Roberts Funds at the Department of Medicine, Weill Cornell Medicine.

**Data sharing statement:** The datasets used and analyzed in this current study are available from the corresponding author on reasonable request.

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**S-Editor:** Wang JJ
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Development and validation of a risk prediction score for the severity of acute hypertriglyceridemic pancreatitis in Chinese patients

Zi-Yu Liu, Lei Tian, Xiang-Yao Sun, Zong-Shi Liu, Li-Jie Hao, Wen-Wen Shen, Yan-Qiu Gao, Hui-Hong Zhai

Abstract

BACKGROUND
The frequency of acute hypertriglyceridemic pancreatitis (AHTGP) is increasing worldwide. AHTGP may be associated with a more severe clinical course and greater mortality than pancreatitis caused by other causes. Early identification of patients with severe inclination is essential for clinical decision-making and improving prognosis. Therefore, we first developed and validated a risk prediction score for the severity of AHTGP in Chinese patients.

AIM
To develop and validate a risk prediction score for the severity of AHTGP in Chinese patients.

METHODS
We performed a retrospective study including 243 patients with AHTGP. Patients were randomly divided into a development cohort (n = 170) and a validation cohort (n = 73). Least absolute shrinkage and selection operator and logistic regression were used to screen 42 potential predictive variables to construct a risk score for the severity of AHTGP. We evaluated the performance of the nomogram and compared it with existing scoring systems. Last, we used the best cutoff value...
(88.16) for severe acute pancreatitis (SAP) to determine the risk stratification classification.

RESULTS
Age, the reduction in apolipoprotein A1 and the presence of pleural effusion were independent risk factors for SAP and were used to construct the nomogram (risk prediction score referred to as AAP). The concordance index of the nomogram in the development and validation groups was 0.930 and 0.928, respectively. Calibration plots demonstrate excellent agreement between the predicted and actual probabilities in SAP patients. The area under the curve of the nomogram (0.929) was better than those of the Bedside Index of Severity in AP (BISAP), Ranson, Acute Physiology and Chronic Health Evaluation (APACHE II), modified computed tomography severity index (MCTSI), and early achievable severity index scores (0.852, 0.825, 0.807, 0.831 and 0.807, respectively). In comparison with these scores, the integrated discrimination improvement and decision curve analysis showed improved accuracy in predicting SAP and better net benefits for clinical decisions. Receiver operating characteristic curve analysis was used to determine risk stratification classification for AHTGP by dividing patients into high-risk and low-risk groups according to the best cutoff value (88.16). The high-risk group (> 88.16) was closely related to the appearance of local and systemic complications, Ranson score ≥ 3, BISAP score ≥ 3, MCTSI score ≥ 4, APACHE II score ≥ 8, C-reactive protein level ≥ 190, and length of hospital stay.

CONCLUSION
The nomogram could help identify AHTGP patients who are likely to develop SAP at an early stage, which is of great value in guiding clinical decisions.

Key Words: Nomogram; Severity; Acute pancreatitis; Prediction model

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prediction is difficult[10]. BISAP uses five indicators to determine AP severity 24 h after admission. It is easy to use but has low sensitivity for predicting SAP[11]. The MCTSI has outstanding performance in predicting local complications. But it is poor in predicting severity[12]. The AP prediction accuracy could be improved by combining several scoring systems, but it was inconvenient. Without a new scoring system, it was difficult to increase the prediction accuracy[13].

Moreover, many studies have demonstrated that single laboratory indicators can be used to predict the severity of AHTGP, such as the neutrophil-lymphocyte ratio (NLR), white blood cell (WBC) count, red blood cell distribution width (RDW), serum calcium (Ca²⁺) and C-reactive protein (CRP), which are easy to use in practice but lack high sensitivity or specificity[2,14]. It should be noted that serum triglyceride (TG) levels dose-dependently worsen the outcome of AP[6], but there is still controversy[5]. Recently, several new clinical prediction models have been developed to predict the severity of AP[15,16]. The early achievable severity index (EASY) prediction score as an artificial intelligence model was recently developed based on machine learning for the early and easy prediction of severity in AP[17]. However, almost all of them were developed for all etiologies of pancreatitis and not for HTG-induced pancreatitis separately.

In conclusion, AHTGP may be associated with a more severe clinical course and greater mortality. Early prediction and detection of patients who are likely to develop SAP is of great importance. The purpose of this study is to develop and validate a fast, simple, accurate, and reproducible risk score for predicting severe AHTGP at an early stage.

### MATERIALS AND METHODS

**Patient selection and data processing**
The study involved a retrospective review of 243 patients diagnosed with AHTGP who were admitted to the Intensive Care Unit of a gastroenterology department of Xuanwu Hospital from November 2012 to January 2022. All patients were diagnosed with AHTGP for the first time, and the possibility of other pancreatic diseases (recurrent AP, chronic pancreatitis, or pancreatic cancer) and cases with missing data were excluded. The following data were recorded: Basic demographics, medical history, vital signs, laboratory tests and X-ray of the chest within 24 h. Pancreatic examinations under CT or magnetic resonance imaging within 72 h. All patients received routine management after admission. This study was approved by the Ethics Committee of the Xuanwu Hospital of Capital Medical University; written informed consent was waived considering the retrospective study design.

**Diagnosis and scoring systems**
The diagnostic criteria for AHTGP were elevated TG level (> 11.30 mmol/L, or 5.65-11.30 mmol/L with lactescent serum) and two or more of the following three symptoms: (1) Abdominal pain consistent with AP; (2) Levels of amylase and/or lipase at least three times above the upper limit of normal; and (3) Abdominal imaging consistent with changes in AP. According to the Atlanta classification revised in 2012, AP severity was divided into three groups based on organ failure status and local and/or systemic complications. The absence of organ failure and local or systemic complications was considered to indicate mild AP (MAP). The presence of transient (within 48 h) organ failure and/or local complications or exacerbation of comorbid disease was regarded as moderately severe AP (MSAP). The presence of persistent (> 48 h) organ failure was considered to indicate SAP. The respiratory, cardiovascular, and renal systems were assessed to identify organ failure, which was defined as a modified Marshall score > 2 for one of these three systems. Acute peripancreatic fluid collection, pancreatic pseudocyst, acute necrotic collection, walled-off necrosis, and infected pancreatic necrosis were defined as local complications[18].

As part of the validation process, five scoring systems were compared to our risk prediction score. Within 24 h of admission, laboratory tests and radiological examinations were conducted to determine APACHE II and BISAP scores. The Ranson score was derived from laboratory tests performed within 48 h of admission. The MCTSI score was determined from CT scans performed within 72 h of admission. The EASY prediction score as an artificial intelligence model was recently developed to predict the severity of AP. It consists of four parts (personal details, anamnestic data, admission data and blood test results) with a total of 23 predictors. We calculated the predicted severity scores for all subjects on a web application (http://easy-app.org/) in the Streamlit Python-based framework.

**Potential predictive variables**
We selected 42 potential predictive variables based on the literature and previous clinical experience, including demographic variables, medical history, clinical signs, laboratory findings, and imaging results. Demographic variables included continuous and categorical variables: Age, body mass index (BMI), smoking status, and drinking habits. Medical history included categorical variables: Diabetes, hypertension, hyperlipemia, and fatty liver. Clinical signs included continuous variables: Respiratory rate (RR), heart rate (HR), systolic blood pressure, and diastolic blood pressure. Laboratory findings performed within 24 h of admission included WBC, NLR[14], RDW[19], platelet counts (PLT), mean
platelet volume (MPV) [20], platelet distribution width (PDW) [21], total cholesterol (TC) [22], TG [6], high-density lipoprotein cholesterol level to low-density lipoprotein cholesterol level ratio (H/L ratio) [23], apolipoprotein A1 (ApoA1) [24], total bilirubin (TBIL), aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB) [25], blood urea nitrogen (BUN) [24], serum creatinine (Cr) [26], free triiodothyronine (fT3) [27], CRP, procalcitonin (PCT), serum sodium (Na), serum potassium (K), serum Ca\(^{2+}\), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (FIB) [28], and D-dimer levels [29]. Imaging results included the presence of a pleural effusion according to the chest X-ray within 24 h of admission [30].

Outcomes
We defined the severity of AHTGP according to the revised Atlanta classification given the extensive acceptance of this guideline [18]. SAP was defined as persistent organ failure and was used as the clinical outcome measure.

Statistical analysis
Statistical comparisons between the non-SAP (MAP + MSAP) and SAP groups were performed with analysis of variance or the Mann–Whitney U test for continuous variables and the chi-square test or Fisher’s exact test for categorical variables. There were 42 variables entered into the selection process, as described here. Least absolute shrinkage and selection operator (LASSO) regression was applied to minimize the potential collinearity of variables measured from the same patient and overfitting of variables. The most predictive covariates were selected by the minimum (\(\lambda\) min). Subsequently, variables identified by LASSO regression analysis and some other important clinical variables were entered into univariable and multivariable logistic regression analyses for further predictor selection. Clinical patient samples were randomly divided (3:1) into development and validation cohorts. We constructed a nomogram and validated the accuracy estimates by using 1000 bootstrap resamples to reduce overfitting. To quantify the discrimination performance of the nomogram for predicting SAP, the concordance index (C-index) was measured in the development set and validation cohort. By plotting the calibration curve, we analyzed the relationship between the observed incidence and predicted probability in the development set and the validation set. Integrated discrimination improvement (IDI) was established to evaluate the improvement of the risk prediction score with other existing scoring systems in the whole set. Decision curve analysis (DCA) was applied to quantify the clinical usefulness of the nomogram in the whole set. All statistical analyses were performed by R software (https://www.r-project.org/, The R Foundation) and Empower-Stats software (http://www.empowerstats.com, X&Y Solutions, Inc., Boston, MA, United States).

Risk group
To determine the risk stratification classification for AHTGP, the best cutoff value for SAP calculated through the ROC curve was used to divide patients into high-risk and low-risk groups. The differences in clinical manifestations and prognoses between the high- and low-risk groups were also compared to evaluate the efficacy of our risk score.

RESULTS

Clinical characteristics
A total of 243 records from patients diagnosed with AHTGP and discharged from the intensive care units (ICUs) during our study period were included for analysis. The baseline characteristics of the study population are summarized in Table 1. The average age of the entire cohort was 40.12 ± 10.12 years, and there were more males (\(n = 188, 77.37\%\)) than females (\(n = 55, 22.63\%\)). We divided our AP patients into two groups, non-SAP (MAP + MSAP) and SAP. The total rate of SAP was 25.51%. There was no significant difference in age, sex or BMI between the two groups. Furthermore, there was no significant difference in medical history between the two groups. In terms of vital signs, patients with SAP had a significantly higher heart rate (92.23 ± 16.29 vs 107.58 ± 18.65, \(P < 0.001\)) and respiratory rate (20.47 ± 4.38 vs 25.27 ± 6.21, \(P < 0.001\)) index than patients in the non-SAP group. Patients in the two groups showed no significant difference in laboratory findings, including PLT, MPV, H/L ratio, TBIL, ALT, ALP, fT3, Na, K, and TT. However, there were differences in WBC, NLR, RDW, PDW, TC, TG, ApoA1, AST, ALB, BUN, Cr, CRP, PCT, ESR, Ca\(^{2+}\), PT, APTT, FIB, and D-dimer. For imaging results, pleural effusions were significantly more frequently observed among SAP patients (12.71% vs 74.19%, \(P < 0.001\)).

Predictor selection
Forty-two variables measured at the hospital within 24 h of admission were included in the LASSO regression. After LASSO regression selection (Figure 1), with a lambda of 0.028, 10 variables remained significant predictors of SAP, including age, HR, RR, ApoA1, WBC, Bun, Cr, Ca\(^{2+}\), D-dimer, and the
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>All (n = 243)</th>
<th>Non-SAP (n = 181)</th>
<th>SAP (n = 62)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.297</td>
</tr>
<tr>
<td>Male</td>
<td>188 (77.37)</td>
<td>143 (79.00)</td>
<td>45 (72.60)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>55 (22.63)</td>
<td>38 (21.00)</td>
<td>17 (27.40)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>40.12 ± 10.12</td>
<td>39.60 ± 10.10</td>
<td>41.60 ± 10.20</td>
<td>0.190</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.27 ± 4.25</td>
<td>28.10 ± 4.00</td>
<td>28.70 ± 4.90</td>
<td>0.367</td>
</tr>
<tr>
<td><strong>Medical history</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.648</td>
</tr>
<tr>
<td>Yes</td>
<td>139 (57.20)</td>
<td>102 (56.40)</td>
<td>37 (59.70)</td>
<td></td>
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<tr>
<td>No</td>
<td>104 (42.80)</td>
<td>79 (43.65)</td>
<td>25 (40.30)</td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.164</td>
</tr>
<tr>
<td>Yes</td>
<td>59 (24.28)</td>
<td>48 (26.50)</td>
<td>11 (17.70)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>184 (75.72)</td>
<td>133 (73.50)</td>
<td>51 (82.30)</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.250</td>
</tr>
<tr>
<td>Yes</td>
<td>152 (62.55)</td>
<td>64 (35.40)</td>
<td>27 (43.50)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>91 (37.45)</td>
<td>64 (35.40)</td>
<td>27 (43.50)</td>
<td></td>
</tr>
<tr>
<td>Fatty liver, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.073</td>
</tr>
<tr>
<td>Yes</td>
<td>185 (76.13)</td>
<td>143 (79.00)</td>
<td>42 (67.70)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>58 (23.87)</td>
<td>38 (21.00)</td>
<td>20 (32.30)</td>
<td></td>
</tr>
<tr>
<td>Drinking, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.483</td>
</tr>
<tr>
<td>Yes</td>
<td>120 (49.38)</td>
<td>87 (48.10)</td>
<td>33 (53.20)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>123 (50.62)</td>
<td>94 (51.90)</td>
<td>29 (46.80)</td>
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<td>Smoking, n (%)</td>
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<td></td>
<td></td>
<td>0.386</td>
</tr>
<tr>
<td>Yes</td>
<td>133 (54.73)</td>
<td>102 (56.40)</td>
<td>31 (50.00)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>110 (45.27)</td>
<td>79 (43.60)</td>
<td>31 (50.00)</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical signs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>96.14 ± 18.17</td>
<td>92.23 ± 16.30</td>
<td>107.58 ± 18.65</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>R (bpm)</td>
<td>21.66 ± 5.33</td>
<td>20.47 ± 4.38</td>
<td>25.27 ± 6.21</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>133.50 ± 17.52</td>
<td>133.51 ± 16.56</td>
<td>133.48 ± 20.21</td>
<td>0.992</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.13 ± 12.03</td>
<td>80.85 ± 11.28</td>
<td>81.92 ± 14.08</td>
<td>0.548</td>
</tr>
<tr>
<td><strong>Laboratory findings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (× 10^9/L)</td>
<td>12.53 ± 3.86</td>
<td>11.96 ± 3.63</td>
<td>14.41 ± 3.97</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NLR</td>
<td>9.94 ± 7.27</td>
<td>8.82 ± 6.98</td>
<td>13.19 ± 7.15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>RDW (fL)</td>
<td>12.87 ± 0.72</td>
<td>12.80 ± 0.69</td>
<td>13.08 ± 0.78</td>
<td>0.009</td>
</tr>
<tr>
<td>PLT (× 10^9/L)</td>
<td>218.91 ± 61.98</td>
<td>216.09 ± 57.99</td>
<td>227.15 ± 72.30</td>
<td>0.226</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>10.75 ± 1.05</td>
<td>10.68 ± 1.01</td>
<td>10.95 ± 1.14</td>
<td>0.074</td>
</tr>
<tr>
<td>PDW (fL)</td>
<td>12.66 ± 2.22</td>
<td>12.47 ± 2.09</td>
<td>13.23 ± 2.47</td>
<td>0.018</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>10.15 ± 4.11</td>
<td>9.54 ± 3.62</td>
<td>11.94 ± 4.92</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>21.08 ± 7.50</td>
<td>20.17 ± 7.31</td>
<td>23.75 ± 7.49</td>
<td>0.001</td>
</tr>
<tr>
<td>H/L ratio</td>
<td>0.96 ± 1.36</td>
<td>0.96 ± 1.36</td>
<td>0.97 ± 1.36</td>
<td>0.946</td>
</tr>
</tbody>
</table>
### Table 1: Comparison of Laboratory Data Between the SAP and Non-SAP Groups

<table>
<thead>
<tr>
<th>Test Item</th>
<th>SAP Group</th>
<th>Non-SAP Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA1 (g/L)</td>
<td>1.02 ± 0.35</td>
<td>1.11 ± 0.34</td>
</tr>
<tr>
<td>TBIL (μmol/L)</td>
<td>14.16 ± 8.29</td>
<td>13.81 ± 7.11</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>29.78 ± 17.41</td>
<td>27.40 ± 13.03</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>31.04 ± 28.70</td>
<td>30.80 ± 29.94</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>73.91 ± 25.30</td>
<td>73.59 ± 22.70</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>41.57 ± 5.91</td>
<td>42.22 ± 5.53</td>
</tr>
<tr>
<td>BUN mmol/L</td>
<td>4.32 ± 2.03</td>
<td>4.05 ± 1.39</td>
</tr>
<tr>
<td>Cr (μmol/L)</td>
<td>62.62 ± 42.98</td>
<td>56.64 ± 15.83</td>
</tr>
<tr>
<td>fT3 (pmol/L)</td>
<td>1.97 ± 0.37</td>
<td>1.99 ± 0.36</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>160.20 ± 127.36</td>
<td>133.55 ± 112.64</td>
</tr>
<tr>
<td>PCT (ng/mL)</td>
<td>0.80 ± 1.93</td>
<td>0.59 ± 1.72</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>42.57 ± 24.76</td>
<td>40.71 ± 25.10</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>133.97 ± 4.29</td>
<td>134.28 ± 4.09</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.02 ± 0.46</td>
<td>3.97 ± 0.35</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>2.13 ± 0.26</td>
<td>2.19 ± 0.18</td>
</tr>
<tr>
<td>PT (s)</td>
<td>13.26 ± 0.98</td>
<td>13.12 ± 0.84</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>37.82 ± 5.28</td>
<td>37.35 ± 5.14</td>
</tr>
<tr>
<td>TT (s)</td>
<td>15.07 ± 1.10</td>
<td>15.08 ± 1.09</td>
</tr>
<tr>
<td>FIB (g/L)</td>
<td>5.70 ± 2.21</td>
<td>5.41 ± 2.10</td>
</tr>
<tr>
<td>D-dimer (μg/L)</td>
<td>1.34 ± 1.83</td>
<td>0.94 ± 0.98</td>
</tr>
</tbody>
</table>

### Imaging results

<table>
<thead>
<tr>
<th>Pleural effusion, n (%)</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>69 (28.40)</td>
<td>174 (71.61)</td>
</tr>
<tr>
<td></td>
<td>(12.71)</td>
<td>(87.30)</td>
</tr>
<tr>
<td></td>
<td>46 (74.19)</td>
<td>16 (25.81)</td>
</tr>
</tbody>
</table>

### Univariable and multivariable logistic regression

Inclusion of these 14 variables in a logistic regression model resulted in four variables that were independently statistically significant predictors of SAP (Table 2): Age (OR: 1.07; 95%CI: 1.01-1.14; \( P = 0.033 \)), ApoA1 (OR: 0.02; 95%CI: 0.00-0.12; \( P < 0.0001 \)), Ca²⁺ (OR: 0.11; 95%CI: 0.01-0.90; \( P = 0.040 \)) and the presence of pleural effusion (OR: 15.61; 95%CI: 5.05-48.24; \( P < 0.0001 \)).

### Construction and validation of the nomogram

Clinical patient samples were randomly divided (3:1) into development and validation cohorts. Due to the small sample size, we used 1000 bootstrap resamples for development and validation to reduce over-the-fit bias. The multivariable analyses demonstrated that age, the reduction in ApoA1 and Ca²⁺, and the presence of pleural effusion were independent risk factors for SAP. Considering that the level of

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BMI: Body mass index; RR: Respiratory rate; HR: Heart rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; SAP: Severe acute pancreatitis; Non-SAP: Mild to moderate acute pancreatitis; WBC: White blood cell; NLR: Neutrophil to lymphocyte ratio; RDW: Red blood cell distribution width; PLT: Platelet counts; MPV: Mean platelet volume; RDW: Platelet distribution width; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; ApoA1: Apolipoprotein A1; TBIL: Total bilirubin; AST: Aspartate transaminase; ALP: Alkaline phosphatase; ALB: Albumin; BUN: Blood urea nitrogen; Cr: Creatinine; fT3: Free triiodothyronine; CRP: C-reactive protein; PCT: Procalcitonin; Na: Sodium; K: Potassium; Ca²⁺: Calcium; PT: Prothrombin time; APTT: Activated partial thromboplastin time; TT: Thrombin time; FIB: Fibrinogen; SAP: Severe acute pancreatitis; Non-SAP: Mild acute pancreatitis and moderately severe acute pancreatitis.

The presence of pleural effusion. According to a previous study, TG levels dose-dependently increase the severity of AP\[6\]. The presence of metabolic syndrome and its components associated with increasing AP severity were also important factors\[31,32\]. Therefore, we included TG level, BMI, history of hypertension and diabetes in the regression analysis. Based on the Endocrine Society Clinical Practice Guideline and previously published study, we divided TG levels into three groups as classified by variables (group 1: 11.3 mmol/L; group 2: 11.3-22.59 mmol/L; group 3: ≥ 22.6 mmol/L) for clinical use\[6\].
Table 2 Univariable and multivariable logistic regression analyses of predictive factors for severe acute pancreatitis in all subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariable regression</th>
<th>Multivariable regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>1.02 (0.99-1.05)</td>
<td>0.190</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.03 (0.96-1.10)</td>
<td>0.566</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.60 (0.29-1.24)</td>
<td>0.167</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.15 (0.64-2.06)</td>
<td>0.648</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>1.05 (1.03-1.07)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>RR (bpm)</td>
<td>1.20 (1.12-1.28)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 11.3</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>11.3-22.6</td>
<td>2.06 (0.57-7.49)</td>
<td>0.273</td>
</tr>
<tr>
<td>≥ 22.6</td>
<td>3.43 (0.96-12.20)</td>
<td>0.057</td>
</tr>
<tr>
<td>ApoA1 (g/L)</td>
<td>0.01 (0.00-0.05)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cr (μmol/L)</td>
<td>1.02 (1.01-1.03)</td>
<td>0.002</td>
</tr>
<tr>
<td>Bun (μmol/L)</td>
<td>1.27 (1.10-1.48)</td>
<td>0.001</td>
</tr>
<tr>
<td>WBC (× 10⁹/L)</td>
<td>1.18 (1.09-1.28)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>0.02 (0.01-0.09)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>D-dimer (μg/L)</td>
<td>1.69 (1.35-2.12)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>19.75 (9.64-40.48)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

BMI: Body mass index; RR: Respiratory rate; HR: Heart rate; SBP: Systolic blood pressure; WBC: White blood cell; ApoA1: Apolipoprotein A1; BUN: Blood urea nitrogen; Cr: Creatinine; Ca: Calcium; SAP: Severe acute pancreatitis; OR: Odds ratio.

TG, BMI and history of hypertension and diabetes may also be important predictors, we constructed three predictive models based on different combinations of these factors. The logistic regression function was as follows: Model 1 (including age, ApoA1, and pleural effusion): Log (odds of SAP) = -0.15 (constant) + 0.06 × age - 5.49 × ApoA1 + 5.37 × (pleural effusion = 1); Model 2 (including age, ApoA1, Ca²⁺ and pleural effusion): Log (odds of SAP) = 4.86 (constant) + 0.06 × age - 5.32 × ApoA1 - 2.40 × Ca²⁺ + 3.13 × (pleural effusion = 1); Model 3 (including age, diabetes, hypertension, BMI, TG, ApoA1 and pleural effusion): Log (odds of SAP) = -7.30 (constant) + 0.09 × age + 0.15 × BMI - 0.35 × (hypertension = 1) - 1.09 × (diabetes = 1) - 0.72 × ApoA1 + 3.98 × (pleural effusion = 1) + 3.39 × (TG = 1) + 3.23 × (TG = 2). ROC curve analysis was applied to evaluate the diagnostic performance of the three predictive models (Supplementary Figure 1). Model 1 had no significant differences in receiver operating characteristic curves (AUCs) and IDI between Model 2 and Model 3 (Supplementary Table 1).

For clinical convenience and easy application, we selected Model 1 with the fewest factors as the final risk prediction score and constructed the nomogram (referred to as AAP) (Figure 2). The C-index for the development and validation cohorts after bootstrapping were 0.930 (95% CI: 0.893-0.967) and 0.928 (95% CI: 0.867-0.990), respectively (Figure 3A and B). This suggested a nomogram with good discrimination. The calibration curve of the nomogram is presented in Figure 3C and D. Calibration plots fall on a 45-degree diagonal line, which shows good agreement between the predicted and actual probabilities in SAP patients in the development and validation cohorts.

**Clinical use**

An ROC curve analysis was applied to evaluate the diagnostic efficacy of the new risk prediction score (referred to as AAP) and other clinical scoring systems, including the BISAP, Ranson, APACHE II, MCTSI and EASY. The AUC values of AAP, BISAP, RASON, APACHE II, MCTSI and EASY to predict SAP were 0.929 (95% CI: 0.889-0.958), 0.852 (95% CI: 0.801-0.894), 0.825 (95% CI: 0.771-0.871), 0.807
Figure 1 Feature selection using the least absolute shrinkage and selection operator binary logistic regression model. A: Least absolute shrinkage and selection operator (LASSO) coefficient profiles of the 42 baseline features; B: Tuning parameter (λ) selection in the LASSO model using 10-fold cross-validation via minimum criteria.

Figure 2 Nomogram for predicting severe acute pancreatitis for patients in the development cohort. SAP: Severe acute pancreatitis.

AAP achieved the highest AUC in predicting SAP among the scoring systems (Table 3). The improvement in the prediction of SAP was evaluated by calculating the IDI. IDIs were employed to compare the discriminative ability between the new model and the other clinical scoring systems. These results demonstrated that our nomogram has a greater potential for accurately predicting SAP than the other four clinical scoring systems (Table 4).

DCA was used to compare the clinical usability and benefits of the nomogram throughout the whole cohort. DCA plots showed that our nomogram had greater net benefits than other system scores for predicting the severity of AP patients, which demonstrated its utility in clinical decision-making (Figure 5).

**Risk stratification based on the Nomogram for SAP**

To determine the risk stratification classification for AP, the best cutoff value (88.16) for SAP calculated through the ROC curve was used to divide patients into high-risk and low-risk groups. We further analyzed the relationship between the AAP cutoff value (88.16) and clinical parameters. The high-risk group was closely related to local and system complications: Ranson ≥ 3, BISAP ≥ 3, MCTSI ≥ 4, APACHE-II ≥ 8, CRP ≥ 190, and the length of hospital stay (Table 5).
DISCUSSION

AHTGP has grown in incidence and importance. According to the previously published literature, HTG is the third most common cause of AP[6]. Clinically, AHTGP is similar to other forms of AP, but it is associated with significantly higher complication rates, severity, and mortality[5,6]. Thus, recognizing risk factors for severe AHTGP in the early stages is very important for triaging patients appropriately to ICUs and providing specific treatments. In the present study, we developed a convenient and specific nomogram based on three predictors for predicting the severity of patients with AHTGP. The nomogram showed great calibration and discriminatory abilities in both the development and validation groups. In addition, our nomogram has shown improved prognostic reliability, accuracy and the best net benefit when compared to other clinical scoring systems, such as BISAP, Ranson, APACHE II, CTSI and an artificial intelligence model, the EASY prediction score. Moreover, the model could distinguish patients into low-risk and high-risk groups according to the best cutoff point (88.16). Patients with higher scores had a higher probability of developing SAP than those with lower scores. The cutoff point can help doctors in making medical decisions.

As mentioned in the introduction, four commonly used AP scoring systems, including APACHE II, Ranson, BISAP, and MCTSI, have limitations on their abilities to identify SAP early. According to our results, our prediction score AAP has three easily available parameters lower than other scoring systems, which is easy for clinical application. Meanwhile, it has better performance in diagnostic efficacy and clinical decision-making for patients with AHTGP. It is worth mentioning that an artificial intelligence model-EASY prediction score consisting of 23 parameters was developed recently based on a multicenter, multinational, prospective and observational study[17]. We applied this model to our research population. Although it achieved a high AUC in predicting SAP, it was still the lowest among the AAP and four commonly used AP scoring systems. This may suggest that the prediction ability of the EASY model is limited for pancreatitis caused by HTG.

Notably, we introduced a new predictor, ApoA1, into our risk score compared with other clinical scoring systems. Recent studies have shown a negative correlation between ApoA1 and the severity of AP, and decreased serum levels of ApoA1 have been linked to the occurrence of SAP in patients[24,33]. The mechanism can be explained as follows: In SAP patients, excessive inflammatory cytokines inhibit synthesis. Recent studies showed a negative correlation between ApoA1 and the severity of AP, and decreased serum levels of ApoA1 have been linked to the occurrence of SAP in patients and lead to lipoprotein degradation[34]. Our previous study showed that the best cutoff point of ApoA1 for
Table 5 The relationship between risk stratification and clinical parameters

<table>
<thead>
<tr>
<th></th>
<th>Low-risk group (n = 158)</th>
<th>High-risk group (n = 85)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Local complications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APFC</td>
<td>43 (27.22%)</td>
<td>68 (80.00%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PPC</td>
<td>3 (1.90%)</td>
<td>10 (11.77%)</td>
<td>0.001</td>
</tr>
<tr>
<td>ANC</td>
<td>3 (1.90%)</td>
<td>22 (25.88%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WON</td>
<td>0 (0.00%)</td>
<td>4 (4.71%)</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Systemic complications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIRS</td>
<td>92 (58.23%)</td>
<td>72 (84.71%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>29 (18.35%)</td>
<td>59 (69.41%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Renal failure</td>
<td>0 (0.00%)</td>
<td>13 (15.29%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cardiovascular failure</td>
<td>2 (1.27%)</td>
<td>5 (5.88%)</td>
<td>0.040</td>
</tr>
<tr>
<td><strong>Clinical Scoring Systems</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranson ≥ 3</td>
<td>28 (17.72%)</td>
<td>53 (62.35%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BISAP ≥ 3</td>
<td>0 (0.00%)</td>
<td>4 (4.71%)</td>
<td>0.014</td>
</tr>
<tr>
<td>MCTSI ≥ 4</td>
<td>15 (9.49%)</td>
<td>53 (62.35%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>APACHE-II ≥ 8</td>
<td>73 (46.20%)</td>
<td>68 (80.00%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP ≥ 190</td>
<td>42 (26.58%)</td>
<td>44 (51.77%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hospital day, days</td>
<td>13.51 ± 5.45</td>
<td>20.04 ± 8.14</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>


predicting severe acute hyperlipidemic pancreatitis was 0.8 g/L, whose sensitivity, specificity and Youden index were 0.877, 0.674 and 0.55, respectively[35]. Another study performed by Li et al[33] showed that ApoA1 predicts the optimal critical value of SAP to be 0.8 g/L, whose sensitivity, specificity and Youden index were 0.551, 0.757 and 0.693, respectively[33]. Therefore, ApoA1 has been found to be a reliable and validated indicator of SAP. Therefore, ApoA1 included in our model could increase the specificity and sensitivity of the nomogram.

Pleural effusion is one of the most common thoracic complications in AP patients. According to recent studies, the prevalence ranges from 46.0% to 72.3%[36]. The disruption of the pancreatic duct may result in leakage of pancreatic secretions directly into the peritoneal cavity via the transdiaphragmatic lymphatic channels[37]. According to the up-to-date revised Atlanta criteria, pleural effusion is a strong individual predictor of SAP. Additionally, pleural effusion is a valuable indicator of BISAP score. Yan et al[30] showed that on the basis of PEV, the AUC of 0.816 for predicting severe AP, with a threshold of 69.00 mL, and the sensitivity and specificity were 84.23% and 81.07, respectively. According to this study, pleural effusion volume can serve as a clinical biomarker to predict the severity and outcome of AP[30].

The evidence indicates that patients with HTG present a more severe form of pancreatitis. A previous study confirmed that HTG dose-dependently increases the complications and severity of AP[6]. However, a meta-analysis including 11965 patients from 16 eligible studies found no significant difference in AP severity based on the extent of HTG[5]. This study also explored the association between HTG levels and the severity of AHTGP. Compared to patients with TGs lower than 11.3 mmol/L, we found that patients with TGs above 22.6 mmol/L had a higher OR (OR: 5.95; 95%CI: 0.56-62.75; P = 0.232) than patients with TGs between 11.3 and 22.59 mmol/L (OR: 4.20; 95%CI: 0.40-44.25; P = 0.138). We speculated that there was a trend for HTG to dose-dependently increase the severity of AHTGP. Previous studies showed that the presence of metabolic syndrome and its components, including obesity, diabetes and hypertension, were significantly associated with increasing AP severity [31,32]. Our study showed that there was a trend that the presence of obesity (OR: 1.08; 95%CI: 0.94-1.24; P = 0.278), diabetes (OR: 1.27; 95%CI: 0.38-4.24; P = 0.693) and hypertension (OR: 1.02; 95%CI: 0.99-1.06; P = 0.201) increased the risk of SAP. However, the P values were not significant, probably because of our small sample size. The small sample size of our study makes it difficult to make extensive recommendations. The next step is to conduct a multicenter prospective cohort study with a large sample size to further explore this important issue.
The novelties and strengths of our study include the following: To the best of our knowledge, this is the first study attempting to develop a risk prediction score for HTG-induced pancreatitis. We also compared the risk prediction score with existing scoring systems in a sample of Chinese patients for predicting the severity of AHTGP. Although the established and validated nomogram in our study may provide a convenient and specific tool to assist physicians in clinical decisions, there are some limitations to be taken into account. First, this study was retrospective, so selection and detection bias could exist. Second, it was a single-center study with a small sample size, which lacked multicenter data verification. There is a need to externally validate the risk score prior to clinical use. Furthermore, the data used to develop and validate the score are all from China, which limits their generalizability to other parts of the world.

**CONCLUSION**

In this study, we developed a risk score to estimate the prediction of developing SAP among patients with AHTGP based on three variables commonly measured on admission to the hospital. Estimating the risk score could help identify patients who are likely to develop SAP at an early stage. It could be of great value in guiding clinical decisions as a convenient and specific tool and optimizing the use of medical resources by supporting appropriate treatment.
Figure 4 Receiver operating characteristic curves for the AAP and other existing clinical scoring systems, such as Bedside Index of Severity in acute pancreatitis, Ranson, Acute Physiology and Chronic Health Evaluation II), modified computed tomography severity index and early achievable severity index prediction scores. AAP: Our risk prediction score referred to as AAP; EASY: The EASY prediction score is an artificial intelligence model for predicting the severity of acute pancreatitis.

Figure 5 Decision curve analysis for the nomogram. AAP: Our risk prediction score referred to as AAP; EASY: The EASY prediction score is an artificial intelligence model for predicting the severity of acute pancreatitis.

ARTICLE HIGHLIGHTS

Research background
The frequency of acute hypertriglyceridemic pancreatitis (AHTGP) is increasing worldwide. AHTGP may be associated with a more severe clinical course and greater mortality than pancreatitis caused by other causes. Early identification of patients with severe inclination is essential for clinical decision-making and improving prognosis. Hence, constructing a risk prediction score with high predictive accuracy and clinical utility for assessing the severity of AHTGP patients is of great importance.

Research motivation
Early prediction and detection of AHTGP patients who are likely to develop severe acute pancreatitis (SAP) is of great importance. Almost of existing clinical scores were developed for all etiologies of pancreatitis and not for hypertriglyceridemia (HTG)-induced pancreatitis separately. To the best of our knowledge, this is the first study attempting to develop a risk prediction score for HTG-induced pancreatitis. This risk score may help guide clinical decisions for these patients.
Research objectives
The purpose of this study was to establish a risk prediction score with easy use and high performance for predicting the severity of AHTGP patients in China, which will help doctors make rational clinical decisions.

Research methods
We performed a retrospective study of patients with AHTGP. Least absolute shrinkage and selection operator and logistic regression were used to screen predictive variables to construct a nomogram for predicting the severity of AHTGP. The predictive accuracy of the nomogram was estimated using the concordance index. The performance of the nomogram was estimated using a calibration curve. We evaluated the predictive accuracy and net benefit of the risk score and compared it with existing scoring systems via receiver operating characteristic curve analysis and decision curve analysis. We used the best cutoff value for SAP to determine the risk stratification classification.

Research results
A risk prediction score consisting of three predictors commonly measured on admission was constructed to predict the severity of SAP. More importantly, our nomogram exhibited high predictive accuracy and good performance. In addition, our nomogram has shown improved prognostic reliability, accuracy and the best net benefit when compared to other clinical scoring systems, such as Bedside Index of Severity in AP, Ranson, Acute Physiology and Chronic Health Evaluation II, modified computed tomography severity index and an artificial intelligence model, the early achievable severity index prediction score. Moreover, the risk prediction score could distinguish patients into low-risk and high-risk groups according to the best cutoff point. The cutoff point can help doctors in making medical decisions.

Research conclusions
This risk prediction score have potential usefulness in predicting the presence of SAP at an early stage. It could be of great value in guiding clinical decisions as a convenient and specific tool and optimizing the use of medical resources by supporting appropriate treatment.

Research perspectives
To the best of our knowledge, this is the first study attempting to develop a risk prediction score for HTG-induced pancreatitis. But, this was a single-center study with a small sample size, which lacked multi-center data verification. The next step is to conduct a multicenter prospective cohort study with a large sample size to construct specific risk score and externally validate the risk score prior to clinical use.

ACKNOWLEDGEMENTS
The authors express special thanks to Gui-Qi Zhu (Department of Liver Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, 200032, China) for his advice on data analysis.

FOOTNOTES
Author contributions: Liu ZY and Zhai HH designed the current study as the principal investigators; Liu ZY and Tian L. were involved in the study conception and design; Hao LJ, Shen WW and Gao YQ collected data; Liu ZY, Sun XY and Liu ZS drafted the plans for the data analyses, conducted statistical analyses and interpreted the data; Liu ZY drafted the manuscript; Tian L was responsible for language editing; All authors were involved in interpretation of the results and revision of the manuscript, and all approved the final version of the manuscript, the corresponding author attests that all the listed authors meet the authorship criteria and that no others meeting the criteria have been omitted.

Supported by 2021 National Natural Youth Cultivation Project of Xuanwu Hospital of Capital Medical University, No. QNPY2021018.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Xuanwu Hospital of Capital Medical University, No. 2022102.

Informed consent statement: Written informed consent was waived considering the retrospective study design.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The clinical data was available from the corresponding author at zhaihuihong@263.net.
no additional data are available.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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Liu ZY et al. Risk score predicting severity of AHTGP


Thong VD, Mon Trinh NT, Phat HT. Factors associated with the severity of hypertriglyceridemia induced acute pancreatitis. Medicine (Baltimore) 2021; 100: e25983 [PMID: 34032712 DOI: 10.1097/MD.00000000000025983]


Are bowel symptoms and psychosocial features different in irritable bowel syndrome patients with abdominal discomfort compared to abdominal pain?

Xiu-Cai Fang, Wen-Juan Fan, Douglas D Drossman, Shao-Mei Han, Mei-Yun Ke

**Abstract**

**BACKGROUND**

The Rome IV criteria eliminated abdominal discomfort for irritable bowel syndrome (IBS), which was previously included in Rome III. There are questions as to whether IBS patients with abdominal discomfort (seen in Rome III but not Rome IV) are different from those with abdominal pain (Rome IV).

**AIM**

To compare bowel symptoms and psychosocial features in IBS patients diagnosed with Rome III criteria with abdominal discomfort, abdominal pain, and pain & discomfort.

**METHODS**
We studied IBS patients meeting Rome III criteria. We administered the IBS symptom questionnaire, psychological status, and IBS quality of life. Patients were classified according to the predominant abdominal symptom associated with defecation into an only pain group, only discomfort group, and pain & discomfort group. We compared bowel symptoms, extraintestinal symptoms, IBS quality of life, psychological status and healthcare-seeking behaviors, and efficacy among the three groups. Finally, we tested risk factors for symptom reporting in IBS patients.

RESULTS
Of the 367 Rome III IBS patients enrolled, 33.8% (124 cases) failed to meet Rome IV criteria for an IBS diagnosis. There were no meaningful differences between the pain group (n = 233) and the discomfort group (n = 83) for the following: (1) Frequency of defecatory abdominal pain or discomfort; (2) Bowel habits; (3) Coexisting extragastrointestinal pain; (4) Comorbid anxiety and depression; and (5) IBS quality of life scores except more patients in the discomfort group reported mild symptom than the pain group (22.9% vs 9.0%). There is a significant tendency for patients to report their defecatory and non-defecatory abdominal symptom as pain alone, or discomfort alone, or pain & discomfort (all \( P < 0.001 \)).

CONCLUSION
IBS patients with abdominal discomfort have similar bowel symptoms and psychosocial features to those with abdominal pain. IBS symptoms manifesting abdominal pain or discomfort may primarily be due to different sensation and reporting experience.

Key Words: Irritable bowel syndrome; Abdominal pain; Abdominal discomfort; Diagnosis; Psychosocial distress; Quality of life

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Core Tip: It is generally accepted that abdominal pain is the most predominant symptom of irritable bowel syndrome (IBS), and Rome IV eliminated abdominal discomfort as diagnostic criteria for IBS. Asian studies showed about one-third of IBS patients diagnosed using Rome III criteria had abdominal discomfort alone. In this study, we compared bowel symptoms, extraintestinal symptoms, IBS-quality of life, psychological status and healthcare-seeking behaviors, and efficacy between the abdominal pain and abdominal discomfort groups expecting to find a difference between the two groups but did not. We also assessed risk factors for symptom reporting for IBS patients.

Citation: Fang XC, Fan WJ, Drossman DD, Han SM, Ke MY. Are bowel symptoms and psychosocial features different in irritable bowel syndrome patients with abdominal discomfort compared to abdominal pain? World J Gastroenterol 2022; 28(33): 4861-4874
URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4861.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4861

INTRODUCTION
Irritable bowel syndrome (IBS) is a common functional bowel disorder with a global prevalence of 4.1% according to the Rome IV criteria and 10.1% with Rome III criteria\[^{1}\]. Using the Rome III definition, IBS is characterized by recurrent abdominal pain or discomfort associated with altered bowel frequency or stool form\[^{2}\]. However, the term “discomfort” was deleted from the 2016 Rome IV diagnostic criteria because some languages do not have a word for discomfort or it has different meanings in different languages or cultures\[^{3,4}\]. Possibly abdominal discomfort has qualitative and quantitative levels of distinction with abdominal pain\[^{5}\]. The data from a population-based survey of adults in the United States, Canada, and the United Kingdom showed that eliminating “discomfort” from the criteria for IBS affected diagnostic rates only slightly\[^{6}\], and only 10% of Rome III-IBS patients among the Swedish cohort did not fulfill Rome-IV IBS diagnosis due to reporting only abdominal discomfort and not pain \[^{7}\]. However, clinical studies from Thailand and central China revealed that about one-third of patients with IBS diagnosed using Rome III criteria had abdominal discomfort alone\[^{8,9}\]. This rate is as high as 84.2% from another clinical retrospective report from Tianjin, China\[^{10}\]. Evidence regarding pathophysiological differences between abdominal pain and abdominal discomfort such as whether these symptoms are categorically different or exist on a continuum of severity is lacking\[^{11,12}\]. It is also unclear whether there are clinical or phenotypical distinctions with IBS presenting with abdominal pain.
vs abdominal discomfort as to how this change of criteria impacts the clinical practice. This study aimed to: (1) Compare the bowel and extraintestinal symptoms of patients with IBS presenting with abdominal discomfort alone to those with pain alone as well as with pain & discomfort; (2) Evaluate the anxiety, depression, quality of life (QOL), and symptom reporting tendency for patients with pain and discomfort; and (3) Validate whether the discomfort is milder than pain on a continuum of severity for Chinese patients. The clinical data were drawn from the IBS database of Peking Union Medical College Hospital.

**MATERIALS AND METHODS**

**Subjects**
Consecutive patients with IBS aged 18-65 years from Peking Union Medical College Hospital gastroenterology clinics were enrolled in this study from June 2009 to February 2016. All patients met Rome III diagnostic and subtype criteria[2], including IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), and mixed IBS. Patients with organic gastrointestinal diseases and metabolic diseases were excluded based on the results of routine tests for blood, urine, stool; liver, kidney, and thyroid function, measurements of carcinoembryonic antigen, erythrocyte sedimentation rate, and C-reactive protein, and abdominal ultrasound and colonoscopy/barium enema in the past year. The participating patients provided oral or written consent to participate before study enrollment. This study was approved by the Peking Union Medical College Hospital Ethics Committee (S-234).

**IBS symptom questionnaire**
The IBS symptom questionnaire was administered by well-trained investigators in face-to-face interviews. The questionnaire was adapted from a previous symptom-related questionnaire for adult functional gastrointestinal disorders in Beijing[13], the Rome III diagnostic questionnaire for adult functional gastrointestinal disorders, and the Rome III psychosocial alarm questionnaire for functional gastrointestinal disorders[2]. Information collected included demographic data, IBS disease course, frequency and severity of IBS symptoms, defecation-related symptoms, extraintestinal symptoms, physical examination and supplementary examination results, and IBS treatments in the whole disease course and the last year.

Patients were evaluated according to abdominal pain, abdominal discomfort, or both abdominal pain & discomfort just before defecation (pre-defecatory), at IBS onset, and between IBS symptom episodes without association to defecation (ordinary). Patients with the presence or worsening of pre-defecatory abdominal pain and without pre-defecatory abdominal discomfort were categorized as the pain group regardless of whether they had abdominal pain or discomfort during the ordinary period. Similarly, patients with pre-defecatory abdominal discomfort and without pre-defecatory abdominal pain were categorized as the discomfort group, and patients with pre-defecatory abdominal pain and discomfort were categorized as the pain & discomfort group.

The main intestinal symptom score for IBS-D was calculated according to the report by Zhu et al[14]. Diagnosis of gastroesophageal reflux disease and functional dyspepsia were made according to the Montreal consensus[15] and Rome III diagnostic and subtype criteria[2], respectively. Patients who did not meet Rome IV diagnostic criteria for IBS (including patients with pre-defecatory abdominal discomfort alone or symptom frequency < 1 d/wk) were evaluated for possible diagnoses of other functional bowel disorders using Rome IV criteria, including functional diarrhea, functional constipation, functional abdominal bloating/distension, and unspecified functional bowel disorder[3].

**QOL evaluation**
The simplified Chinese version of the IBS-QOL instrument was used to evaluate patient QOL[16], which was translated from IBS-QOL[17] and well validated. This instrument was completed by patients according to the instructions provided; the total score and eight domain scores were calculated as in a previous publication[14].

**Psychological evaluation**
The Hamilton Anxiety (HAMA) and Hamilton Depression (HAMD) scales were used to evaluate patient psychological status by specially trained professionals through conversation and observation. A HAMA score ≥ 14 was judged as anxiety and ≥ 21 as moderate-to-severe anxiety. A HAMD score ≥ 17 was judged as depression and ≥ 24 as moderate-to-severe depression[18,19].

**Statistical analysis**
All analyses were performed using SPSS version 19.0 (IBM Corporation, Somers, NY, United States). Parametric distribution was evaluated by Kolmogorov-Smirnov test. Parametric and categorical data are presented as mean ± SD or rate, respectively. Nonparametric data were presented as median and interquartile range. Comparisons among the three groups were made by one-way analysis of variance.
for parametric data, Kruskal-Wallis test for nonparametric data, and χ² test for categorical variables. Spearman’s test was performed to assess nonparametric correlations between two quantitative variables. Bonferroni test was used to adjust for pairwise comparison among the three groups after analysis of variance. Multiple logistic regression analysis was used to determine the independent factors for abdominal pain or abdominal discomfort. P < 0.05 was considered statistically significant.

RESULTS

Demographic data
In total, 367 patients meeting Rome III criteria for IBS were enrolled in this study (205 males and 162 females), with an average age of 43.0 ± 11.4 years. There were 233 patients (63.5%) in the pain group, 83 patients (22.6%) in the discomfort group, and 51 patients (13.9%) in the pain & discomfort group. There were more males in the discomfort group than in the pain group (67.5% vs 50.2%, P = 0.01). There were no significant differences in age, body mass index, educational level, physical work, family economic status, marriage status, the average IBS disease course, and IBS subtype distribution among the three groups (P > 0.05) (Table 1).

Characteristics of abdominal pain, discomfort, and pain & discomfort
In the three groups, the locations of abdominal pain, discomfort, or pain & discomfort before defecation were mainly in the umbilical region, lower abdomen, and left lower quadrant. There was no significant difference in distribution of symptom location, even though more patients in the discomfort group reported the symptom location as “others” (indicating varied or obscure locations) than in the pain group (21.7% vs 10.3%, P = 0.009). There was a significant difference in the severity of pain and/or discomfort among the three groups (P = 0.007), and more patients in the discomfort group reported mild symptom than those in the pain group. There was no significant difference in frequency among the three groups (Table 2).

There were significant differences in the prevalence of ordinary abdominal pain or/and discomfort among the three groups (P < 0.001). More patients in the pain group reported ordinary abdominal pain than those in the discomfort group and pain & discomfort group, while more patients in the discomfort group reported ordinary abdominal discomfort than those in the pain group and pain & discomfort group. In the pain & discomfort group, 54.9% of patients reported having ordinary abdominal pain and discomfort, which was significantly higher than the other two groups (Table 2).

In total, there were 52 patients (14.2%) with onset frequency of < 1 d/wk (i.e. 3 d/mo), including 37 cases in the pain group, 11 cases in the discomfort group, and 4 cases in the pain & discomfort group. The proportion of less frequency was 15.9%, 13.3%, and 7.8%, respectively, without significant difference (P = 0.32). According to Rome IV diagnostic criteria, a total of 124 patients (33.8%) would not meet an IBS diagnosis (Figure 1).

Bowel movements and stool form
In 345 patients with IBS-D, the average bowel movements during symptom non-onset period of the pain group (1.5 ± 0.9/d) were less than the discomfort group (1.8 ± 1.1/d) and the pain & discomfort group (1.9 ± 1.1/d) (P = 0.004), but there were no significant differences in average bowel movements during symptom onset period (3.8 ± 1.5 vs 3.8 ± 1.4 vs 3.6 ± 1.5, P > 0.05) (Figure 2A). There were no significant differences in stool form during symptom non-onset and onset periods among the three groups (all P > 0.05) (Figure 2B).

Abdominal pain and/or discomfort improvement after defecation
Abdominal pain and/or discomfort improved after defecation except for 1 patient in the pain group. There was no significant difference in the waiting time and degree for improvement among the three groups (Figure 2C and D).

In IBS-D patients, the main intestinal symptom score was 9.3 ± 1.6 in the pain group, 9.4 ± 1.5 in the discomfort group, and 9.6 ± 1.3 in the pain & discomfort group (P > 0.05).

Defecation-related symptoms
The prevalence of defecation related symptoms such as abdominal bloating, urgency, sensation of incomplete evacuation, and passing mucus were high overall for all 3 groups. More patients in the discomfort group reported having urgency, sensation of incomplete evacuation, and passing mucus than those in the pain group (all P < 0.05). In the pain & discomfort group, the prevalence of abdominal bloating, abdominal distension, and anorectal pain was significantly higher than that in the pain group (all P < 0.05) (Table 2).

Extraintestinal symptoms
There were no significant differences in the prevalence of gastroesophageal reflux disease or functional
Table 1 Demographic data for irritable bowel syndrome patients with abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pain group (n = 233)</th>
<th>Discomfort group (n = 83)</th>
<th>Pain &amp; discomfort group (n = 51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, %</td>
<td>117 (50.2)</td>
<td>56 (67.5)</td>
<td>32 (62.7)</td>
<td>0.012</td>
</tr>
<tr>
<td>Age in yr</td>
<td>43.7 ± 11.7</td>
<td>42.3 ± 10.6</td>
<td>40.8 ± 11.0</td>
<td>0.23</td>
</tr>
<tr>
<td>BMI in kg/m²</td>
<td>23.0 ± 4.0</td>
<td>22.8 ± 4.0</td>
<td>22.3 ± 3.8</td>
<td>0.56</td>
</tr>
<tr>
<td>Education level, college and above, %</td>
<td>71 (30.5)</td>
<td>29 (34.9)</td>
<td>13 (25.5)</td>
<td>0.51</td>
</tr>
<tr>
<td>Physical labor, %</td>
<td>135 (57.9)</td>
<td>42 (50.6)</td>
<td>34 (66.7)</td>
<td>0.18</td>
</tr>
<tr>
<td>Family economic status, well-off &amp; above, %</td>
<td>105 (45.1)</td>
<td>44 (53.0)</td>
<td>18 (35.3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Marriage status, married, %</td>
<td>201 (86.3)</td>
<td>71 (85.5)</td>
<td>41 (80.4)</td>
<td>0.56</td>
</tr>
<tr>
<td>IBS disease course in yr¹</td>
<td>6.0 (7.5)</td>
<td>5.3 (7.0)</td>
<td>6.0 (7.0)</td>
<td>0.38</td>
</tr>
<tr>
<td>IBS type</td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>IBS-D, %</td>
<td>95.7</td>
<td>96.4</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td>IBS-C, %</td>
<td>3.0</td>
<td>2.4</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>IBS-M, %</td>
<td>1.3</td>
<td>1.2</td>
<td>5.9</td>
<td></td>
</tr>
</tbody>
</table>

¹Data presented as median (interquartile range), Kruskal-Wallis test. Note: P value is the difference among pain group, discomfort group, and pain & discomfort group, superscript letter is significantly different at a P < 0.05.

Comorbid anxiety and depression

There were no significant differences in HAMA score, HAMD score, or the prevalence and severity of anxiety and depression among the three groups (Table 4).

IBS-QOL

The QOL of patients with IBS showed an obvious decrease with an IBS-QOL score of 72.2 ± 17.9 in the
Table 2 Characteristics of bowel symptoms in irritable bowel syndrome patients with abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pain group (n = 233)</th>
<th>Discomfort group (n = 83)</th>
<th>Pain &amp; discomfort group (n = 51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location, %</td>
<td></td>
<td></td>
<td></td>
<td>0.213</td>
</tr>
<tr>
<td>Left lower quadrant</td>
<td>67 (28.8)</td>
<td>14 (16.9)</td>
<td>12 (23.5)</td>
<td></td>
</tr>
<tr>
<td>Umbilical</td>
<td>79 (33.9)</td>
<td>27 (32.5)</td>
<td>20 (39.2)</td>
<td></td>
</tr>
<tr>
<td>Lower abdomen</td>
<td>65 (27.9)</td>
<td>23 (27.7)</td>
<td>15 (29.4)</td>
<td></td>
</tr>
<tr>
<td>Epigastric</td>
<td>11 (4.7)</td>
<td>2 (2.4)</td>
<td>2 (3.9)</td>
<td></td>
</tr>
<tr>
<td>Whole abdomen</td>
<td>12 (4.0)</td>
<td>8 (9.6)</td>
<td>5 (9.8)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>24 (10.3)</td>
<td>18 (21.7)</td>
<td>7 (13.7)</td>
<td></td>
</tr>
<tr>
<td>Severity, %</td>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>Mild</td>
<td>21 (9.0)</td>
<td>19 (22.9)</td>
<td>6 (11.7)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>160 (68.7)</td>
<td>55 (66.3)</td>
<td>37 (72.6)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>52 (22.3)</td>
<td>9 (10.8)</td>
<td>8 (15.7)</td>
<td></td>
</tr>
<tr>
<td>Frequency, %</td>
<td></td>
<td></td>
<td></td>
<td>0.290</td>
</tr>
<tr>
<td>3 d/mo</td>
<td>37 (15.9)</td>
<td>11 (13.3)</td>
<td>4 (7.84)</td>
<td></td>
</tr>
<tr>
<td>1 d/wk</td>
<td>25 (10.7)</td>
<td>5 (6.0)</td>
<td>2 (3.9)</td>
<td></td>
</tr>
<tr>
<td>&gt;1 d/wk</td>
<td>108 (46.4)</td>
<td>38 (45.8)</td>
<td>27 (52.94)</td>
<td></td>
</tr>
<tr>
<td>Every day</td>
<td>63 (27.0)</td>
<td>29 (34.9)</td>
<td>18 (35.3)</td>
<td></td>
</tr>
<tr>
<td>Ordinary pain/discomfort, %</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pain alone</td>
<td>84 (36.1)</td>
<td>6 (7.2)</td>
<td>6 (11.8)</td>
<td></td>
</tr>
<tr>
<td>Discomfort alone</td>
<td>21 (9.0)</td>
<td>43 (51.8)</td>
<td>3 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Pain &amp; discomfort</td>
<td>7 (3.0)</td>
<td>2 (2.4)</td>
<td>28 (54.9)</td>
<td></td>
</tr>
<tr>
<td>No pain or discomfort</td>
<td>121 (51.9)</td>
<td>32 (38.6)</td>
<td>14 (27.4)</td>
<td></td>
</tr>
<tr>
<td>Defecation-related symptoms, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal bloating</td>
<td>93 (39.9)</td>
<td>43 (51.8)</td>
<td>35 (68.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Abdominal distension</td>
<td>21 (9.0)</td>
<td>13 (15.7)</td>
<td>12 (23.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Urgency</td>
<td>197 (84.6)</td>
<td>80 (96.4)</td>
<td>42 (82.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Defecation straining</td>
<td>70 (30.0)</td>
<td>25 (30.1)</td>
<td>23 (45.1)</td>
<td>0.10</td>
</tr>
<tr>
<td>Sensation of anorectal obstruction</td>
<td>62 (26.6)</td>
<td>30 (36.1)</td>
<td>19 (37.3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Anorectal pain</td>
<td>28 (12.0)</td>
<td>15 (18.1)</td>
<td>17 (33.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sensation of incomplete evacuation</td>
<td>164 (70.4)</td>
<td>74 (89.2)</td>
<td>39 (76.5)</td>
<td>0.003</td>
</tr>
<tr>
<td>Passing mucus</td>
<td>141 (60.5)</td>
<td>66 (79.5)</td>
<td>39 (76.5)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Some patients reported more than one location. χ² test, data presented as number (%).

The difference is between pain group and discomfort group.

The difference is between pain group and pain & discomfort group.

The difference is between discomfort group and pain & discomfort group.

P value is the difference among pain group, discomfort group, and pain & discomfort group, and superscript letters are significantly different at a P < 0.05.

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pain group, 72.0 ± 20.0 in the discomfort group, and 70.4 ± 15.0 in the pain & discomfort group while comparing to the mean overall score in healthy Chinese subjects (95.50 ± 6.73 with the scores on each of the eight domains being ≥ 90.00)[16]. The most meaningful impairment for all 3 groups was food avoidance, following by dysphoria, interference with activity, and health worry. There were no significant differences in the eight domain scores between the pain group and discomfort group (Figure 3), while patients in the pain & discomfort group had lower QOL than patients having discomfort alone (P = 0.03).
Table 3 Coexisting extraintestinal symptoms of irritable bowel syndrome patients with abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pain group (n = 233)</th>
<th>Discomfort group (n = 83)</th>
<th>Pain &amp; discomfort group (n = 51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GERD, %</td>
<td>60 (25.8)</td>
<td>14 (16.9)</td>
<td>10 (19.6)</td>
<td>0.20</td>
</tr>
<tr>
<td>Heartburn</td>
<td>35 (15.0)</td>
<td>6 (7.2)</td>
<td>6 (11.8)</td>
<td>0.18</td>
</tr>
<tr>
<td>Acid reflux</td>
<td>44 (18.9)</td>
<td>10 (12.1)</td>
<td>5 (9.8)</td>
<td>0.15</td>
</tr>
<tr>
<td>Food regurgitation</td>
<td>14 (6.0)</td>
<td>4 (4.8)</td>
<td>3 (5.9)</td>
<td>0.92</td>
</tr>
<tr>
<td>Retrosternal chest pain</td>
<td>10 (4.3)</td>
<td>3 (3.6)</td>
<td>2 (3.9)</td>
<td>0.96</td>
</tr>
<tr>
<td>Functional dyspepsia, %</td>
<td>86 (36.9)</td>
<td>23 (27.7)</td>
<td>18 (35.3)</td>
<td>0.32</td>
</tr>
<tr>
<td>Epigastric pain syndrome</td>
<td>49 (21.0)</td>
<td>6 (7.2)</td>
<td>7 (13.7)</td>
<td>0.01(^2)</td>
</tr>
<tr>
<td>Epigastric pain</td>
<td>43 (18.5)</td>
<td>5 (6.0)</td>
<td>7 (13.7)</td>
<td>0.02(^2)</td>
</tr>
<tr>
<td>Epigastric burning</td>
<td>12 (5.2)</td>
<td>2 (2.4)</td>
<td>3 (5.9)</td>
<td>0.54</td>
</tr>
<tr>
<td>Postprandial distress syndrome</td>
<td>64 (27.5)</td>
<td>22 (26.5)</td>
<td>15 (29.4)</td>
<td>0.94</td>
</tr>
<tr>
<td>Postprandial fullness</td>
<td>57 (24.5)</td>
<td>20 (24.1)</td>
<td>9 (17.7)</td>
<td>0.57</td>
</tr>
<tr>
<td>Early satiation</td>
<td>14 (6.0)</td>
<td>6 (7.2)</td>
<td>9 (17.7)</td>
<td>0.02(^2)</td>
</tr>
<tr>
<td>Somatic pain, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>17 (45.9)</td>
<td>37 (44.6)</td>
<td>26 (51.0)</td>
<td>0.76</td>
</tr>
<tr>
<td>Neck pain</td>
<td>21 (9.0)</td>
<td>7 (8.4)</td>
<td>3 (5.9)</td>
<td>0.77</td>
</tr>
<tr>
<td>Backache</td>
<td>41 (17.6)</td>
<td>8 (9.6)</td>
<td>7 (13.7)</td>
<td>0.21</td>
</tr>
<tr>
<td>Dyspareunia</td>
<td>12 (5.2)</td>
<td>6 (7.2)</td>
<td>11 (21.6)</td>
<td>&lt; 0.001(^3,4)</td>
</tr>
<tr>
<td>Menstrual pain(^1)</td>
<td>30 (25.9)</td>
<td>10 (37.0)</td>
<td>11 (57.9)</td>
<td>0.016(^3)</td>
</tr>
</tbody>
</table>

\(^1\)The number of female patients in the abdominal pain, abdominal discomfort, and pain & discomfort groups was 116, 27, and 19, respectively. \(^2\)P value is difference between the pain group and the discomfort group. \(^3\)The difference is between the pain group and the pain & discomfort group. \(^4\)The difference is between the discomfort group and the pain & discomfort group. \(^5\)P value is the difference among the pain group, discomfort group, and pain & discomfort group, and superscript letters are significantly different at P < 0.05. GERD: Gastroesophageal reflux disease.

**Healthcare-seeking behaviors and efficacy**

There were no significant differences among the three groups in the average number of consultations and colonoscopies in the whole disease course and the average consultations and intermittent and long-term medication use in the last year (all P > 0.05). More patients in discomfort group used antispasmodics (muscarinic cholinergic receptor antagonists and selective intestinal calcium channel blockers), and all patients who used the antispasmodics had a reasonably good response (response rate over 50%). The overall satisfaction rate (including complete satisfaction and satisfaction) with medical care showed no significant difference among the three groups (P > 0.05) (Table 5).

**Risk factors for IBS patients describing pre-defecatory symptoms as abdominal pain alone, discomfort alone, and pain & discomfort**

Twelve variables differing between the pain group and the discomfort group at a P value with significant difference in Tables 1-3 were utilized for a multiple logistic regression analysis. We found that male patients [odds ratio (OR) = 1.955, 95% confidence interval (CI): 1.104-3.462, P = 0.021] and patients with mild defecatory abdominal pain or discomfort [OR = 4.020, 95%CI: 1.436-11.253, P = 0.008] were the predictors for patients to describe their pre-defecatory symptoms as abdominal discomfort alone rather than abdominal pain alone (Table 6). Similar analyses were performed between the pain group and the pain & discomfort group (11 variables) and the discomfort group and the pain & discomfort group (10 variables). We found that abdominal bloating [OR = 2.238, 95%CI: 1.080-4.638, P = 0.030] and anorectal pain [OR = 2.979, 95%CI: 1.347-6.585, P = 0.007] were the predictors for patients to describe their symptom as pain & discomfort rather than pain alone (Table 6), and no predictors were found for patients to describe their symptom as discomfort alone or pain & discomfort.
Table 4 Comorbid anxiety and depression among irritable bowel syndrome patients with abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pain group (n = 233)</th>
<th>Discomfort group (n = 83)</th>
<th>Pain &amp; discomfort group (n = 51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMA score</td>
<td>16.1 ± 7.3</td>
<td>15.5 ± 7.3</td>
<td>17.3 ± 7.4</td>
<td>0.36</td>
</tr>
<tr>
<td>Comorbid anxiety, %</td>
<td>141 (60.5)</td>
<td>49 (59.0)</td>
<td>38 (74.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>Mild</td>
<td>69 (29.6)</td>
<td>25 (30.1)</td>
<td>21 (41.2)</td>
<td>0.26</td>
</tr>
<tr>
<td>Moderate-severe</td>
<td>72 (30.9)</td>
<td>24 (28.9)</td>
<td>17 (33.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>HAMD score</td>
<td>13.2 ± 6.2</td>
<td>12.3 ± 6.1</td>
<td>14.3 ± 5.5</td>
<td>0.18</td>
</tr>
<tr>
<td>Comorbid depression, %</td>
<td>66 (28.3)</td>
<td>22 (26.5)</td>
<td>18 (35.3)</td>
<td>0.53</td>
</tr>
<tr>
<td>Mild</td>
<td>54 (23.2)</td>
<td>20 (24.1)</td>
<td>17 (33.3)</td>
<td>0.31</td>
</tr>
<tr>
<td>Moderate-severe</td>
<td>12 (5.2)</td>
<td>2 (2.4)</td>
<td>1 (2.0)</td>
<td>0.40</td>
</tr>
<tr>
<td>Comorbid anxiety &amp; depression, %</td>
<td>62 (26.6)</td>
<td>20 (24.1)</td>
<td>18 (35.3)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD or number (%). Student’s t test and χ² tests. P value is difference among the pain group, discomfort group, and pain & discomfort group. HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale.

Table 5 Consultations and medications of irritable bowel syndrome patients with abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pain group (n = 233)</th>
<th>Discomfort group (n = 83)</th>
<th>Pain &amp; discomfort group (n = 51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consultation times per year 1</td>
<td>4.6 ± 6.7</td>
<td>5.7 ± 6.2</td>
<td>4.2 ± 4.1</td>
<td>0.54</td>
</tr>
<tr>
<td>Colonoscopies 2</td>
<td>1.9 ± 1.4</td>
<td>1.5 ± 0.8</td>
<td>1.6 ± 0.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Consultation times 1</td>
<td>4.0 ± 5.7</td>
<td>4.5 ± 4.5</td>
<td>4.9 ± 4.7</td>
<td>0.54</td>
</tr>
<tr>
<td>Medications, intermittent and long-term use, %</td>
<td>164 (70.4)</td>
<td>56 (67.5)</td>
<td>43 (84.3)</td>
<td>0.09</td>
</tr>
<tr>
<td>Use rate</td>
<td>29 (12.4)</td>
<td>24 (28.9)</td>
<td>12 (23.5)</td>
<td>0.002³</td>
</tr>
<tr>
<td>Response rate</td>
<td>22 (75.9)</td>
<td>13 (54.2)</td>
<td>10 (83.3)</td>
<td>0.12</td>
</tr>
<tr>
<td>Overall satisfaction to medical care, %</td>
<td>125 (53.7)</td>
<td>39 (47.0)</td>
<td>21 (41.2)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

¹Consultation times were average consultation times of consulters.
²Colonoscopies were average colonoscopies of patients who performed colonoscopies.
³The difference is between the pain group and the discomfort group.

Data presented as mean ± SD or number (%). Analysis of variance and χ² test. P value is difference among pain group, discomfort group, and pain & discomfort group, and superscript letter is significantly different at a P < 0.05.

Diagnosis of patients with abdominal discomfort alone according to Rome IV criteria

Among 83 patients having pre-defecatory abdominal discomfort alone and not meeting Rome IV criteria for IBS, 48 patients (57.8%) met the diagnosis for functional diarrhea, 28 patients (33.7%) for functional abdominal bloating/distension, 2 patients (2.4%) for functional constipation, and 5 patients (6.0%) were classified as unspecified functional bowel disorder.

DISCUSSION

The present study comprehensively compared the bowel symptoms and psychosocial features of IBS
Table 6 Risk factors for irritable bowel syndrome patients describing symptoms as abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort

<table>
<thead>
<tr>
<th>Partial regression coefficient</th>
<th>Standard error</th>
<th>Wald χ²</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal discomfort alone vs abdominal pain alone</td>
<td>Male sex</td>
<td>0.671</td>
<td>0.291</td>
<td>5.293</td>
</tr>
<tr>
<td>Abdominal pain alone vs abdominal pain &amp; discomfort</td>
<td>Severity (mild defecatory pain or discomfort)</td>
<td>1.391</td>
<td>0.525</td>
<td>7.018</td>
</tr>
<tr>
<td>Abdominal bloating</td>
<td>Abdominal bloating</td>
<td>0.805</td>
<td>0.372</td>
<td>4.692</td>
</tr>
<tr>
<td>Anorectal pain</td>
<td>Anorectal pain</td>
<td>1.091</td>
<td>0.405</td>
<td>7.272</td>
</tr>
</tbody>
</table>

*P < 0.05. Multiple logistic regression analysis. Superscript letter is significantly different at a P < 0.05. CI: Confidence interval.

Figure 2 Comparison of bowel movements and stool forms in irritable bowel syndrome with diarrhea patients and improvement of abdominal pain or discomfort after defecation in irritable bowel syndrome patients among the abdominal pain alone, abdominal discomfort alone, and abdominal pain & discomfort groups. A: Bowel movements during irritable bowel syndrome with diarrhea non-onset and onset status; B: Stool forms based on Bristol Stool Form Scale during irritable bowel syndrome with diarrhea non-onset and onset status; C: Degree of improvement of abdominal pain and discomfort with defecation; D: Waiting time for improvement of abdominal pain and discomfort with defecation in irritable bowel syndrome patients. Numbers in the column are percentages. *P < 0.01. BM: Bowel movement.

patients with pre-defecatory abdominal pain alone to pre-defecatory abdominal discomfort alone, and abdominal pain & discomfort. We found that patients with abdominal discomfort had similar bowel and extraintestinal symptoms, comorbid anxiety and depression, QOL, and healthcare-seeking behaviors to those with abdominal pain.

It is generally accepted that abdominal pain is the most predominant symptom of IBS[3]; however, a previous clinical study from the United States found only 21% of IBS patients with moderate to severe symptoms reported their predominant symptom in terms of abdominal pain[11]. Another study conducted by Lembo et al[12] showed that the proportions of IBS patients who reported pain or gas...
Figure 3 Comparison of irritable bowel syndrome-quality of life. There were no significant differences in the total score and eight domain scores among the three groups. Numbers in the column are percentages. IBS-QOL: Irritable bowel syndrome-quality of life.

(bloating-type discomfort) as one of their viscerosensory symptoms were similar (60% vs 66%).

Currently, several studies compared the diagnostic rate between Rome III and IV criteria for IBS in the general population and consulting cohorts. The proportions of having abdominal discomfort varied among the western countries (2.4%-9.9%) [6,7,20,21] and the eastern countries (29.8%-84.2%) [8-10]. In this study, IBS patients with abdominal discomfort accounted for 22.6%. The elimination of abdominal discomfort from the diagnostic criteria had little effect on the diagnosis of IBS for the western countries [3], while a significant proportion of IBS patients were no longer IBS in Asian, including in China [8-10].

The significant difference between the western and eastern countries indicates there may be cultural factors that affect the experience and reporting of abdominal symptoms. The definition of abdominal pain is more uniformly accepted, while the definition for abdominal discomfort is ambiguous; "discomfort means an uncomfortable sensation not described as pain" according to the Rome III criteria [2]. Further, there are no comparison studies concerning abdominal discomfort descriptions in cross-cultural cohorts. In this study, Chinese patients with IBS accurately reported abdominal discomfort, including the location and association with defeaction (both in pre-defecatory and non-defecatory periods), as well as other defection related symptoms (i.e. urgency and so on). Symptom characteristics were similar with abdominal pain, which indicated that abdominal discomfort was a relatively explicit symptom for Chinese patients, unlike the impression from a cognitive study from American IBS patients [6] in which abdominal discomfort might encompass a wide range of symptoms such as bloating, gas, fullness, flatulence, sensation of incomplete evacuation, and urgency.

Abdominal pain and discomfort are both visceral perceptions of abnormality on the same continuum with pain appearing at the more severe end of the spectrum [11]. In this study, there were no meaningful differences between the pain alone group and discomfort alone group in frequencies as well as the main intestinal symptom score for IBS-D patients except more patients in the discomfort group reported mild symptoms than the pain group. In addition, we found patients with mild defeactory abdominal pain or discomfort were predisposed to describe their pre-defecatory symptoms as abdominal discomfort alone rather than abdominal pain alone, which indicated abdominal discomfort may appear as the milder form of pain. However, it was reported that more IBS patients rank abdominal discomfort as their most bothersome symptom than abdominal pain (60% vs 29% in America [12], 15.3% vs 4.5% of IBS-C in Japan [22]), and the severity of abdominal discomfort had the strongest independent relationship with QOL impairment [10]. Patients in the three groups had similar healthcare-seeking behavior and satisfaction to medical care in this study. We speculated in terms of the symptom itself, the overall severity of IBS, and occupation of medical resources that abdominal discomfort is as important as abdominal pain.

Nevertheless, more patients in the discomfort group reported accompanying urgency, sensation of incomplete evacuation, and passing mucus than the pain group. Patients with abdominal pain & discomfort had a higher prevalence of abdominal bloating/distension and anorectal pain than patients with abdominal pain alone, and a lower score of QOL than patients with abdominal discomfort alone. In addition, we found that abdominal bloating and anorectal pain were the predictors for patients to describe their symptom as pain & discomfort rather than pain alone, suggesting coexisting symptoms played important roles in the generation of discomfort feeling.

We noticed that the previous studies seldom paid attention to the abdominal symptoms of IBS patients during non-defecatory period. An interesting finding in this study is more patients having pre-defecatory abdominal discomfort alone also reported non-defecatory abdominal discomfort than the other two groups, and a similar report tendency for patients with pain alone and pain & discomfort
during defecatory period and non-defecatory period. In terms of extraintestinal symptoms, more patients in the pain group reported coexisting epigastric pain. The possible explanation for this reporting tendency is individual sensation and reporting experience to the similar stimulations and pathophysiological changes[11].

The relationships between diary stress, psychological distress, and severity of abdominal discomfort symptoms in women with IBS have been noted[23]. In this study, the scores of HAMA and HAMD and comorbid anxiety and depression were comparable between the pain group and the discomfort group. The impact of mental status to the symptom sensation and reporting could be ignored.

To date, studies on the pathophysiology of IBS mainly focused on abdominal pain[12,24-27]. As far as we know, there was no direct evidence focused on mechanism of abdominal discomfort or comparison of the difference of pathogenesis between abdominal pain and discomfort. Abdominal discomfort could simultaneously improve with abdominal pain and/or bloating to antispasmodics tiapropramide and octylonium, secretagogue linaclotide, or simethicone and Bacillus coagulans for IBS or IBS-C patients[28-31]. It is unclear whether the treatments focused on bloating, diarrhea, or constipation could relieve the abdominal discomfort for those patients having defecatory abdominal discomfort alone while they are diagnosed as other bowel disorders according to Rome IV criteria (as shown in the results). Therefore, we realized that it may be more beneficial to classify patients with bowel-related abdominal discomfort into IBS from a therapeutic consideration.

There are several limitations in this study. We only included the IBS patients with typical changes of bowel habits, i.e. IBS-D and IBS-C. Therefore, some mixed IBS and IBS-unclassified patients might be missed[7,31]. We enrolled patients with Rome III criteria and did not concern the abdominal pain and discomfort during or soon after bowel movement. The proportion of Rome III suspected IBS patients with this kind of pain or discomfort was low (2.9% according to Bai et al[32]). Moreover, we did not ask patients to describe the difference between abdominal pain and discomfort. The data for response to therapies were retrospective recall, including prescription and over-the-counter. In addition, the prevalence of IBS in the general population for males was lower than females (4.1% vs 5.4%)[32], but an equal or higher ratio of male to female consulting patients was reported in clinical studies[9,14]. It is unclear whether male patients have more vigorous healthcare seeking behaviors or priority of medical care than female patients, but more female patients reported frequent consultations and colonoscopies during the whole disease course of IBS than male patients[33]. IBS-D is the predominant subtype, which accounted for 74.1% in the general population of South China[34] and 66.3% in consulting patients[31]. In addition, this was a single-center study.

CONCLUSION

Chinese patients with IBS can differentiate and report abdominal pain or/and abdominal discomfort as their key bowel symptom. The patients with abdominal discomfort had similar bowel symptoms and psychosocial features to those with abdominal pain. There is a tendency for IBS patients to report their defecatory and non-defecatory abdominal symptom as pain alone, discomfort alone, or pain and discomfort. Pre-defecatory abdominal discomfort should be considered as an important symptom for IBS patients. Further studies focused on the pathophysiology and therapeutic response (including the cultural influence) of abdominal pain and discomfort are needed.

ARTICLE HIGHLIGHTS

Research background
The Rome IV criteria eliminated abdominal discomfort for irritable bowel syndrome (IBS), which was previously included in the Rome III criteria. Asian studies showed the rate of IBS patients with abdominal discomfort alone was high.

Research motivation
There are questions as to whether IBS patients with abdominal discomfort (seen in Rome III but not Rome IV) are different from those with abdominal pain (Rome IV).

Research objectives
To compare the bowel and extraintestinal symptoms of patients with IBS presenting with abdominal discomfort alone to those with pain alone as well as with pain & discomfort and to evaluate the anxiety, depression, quality of life, and symptom reporting tendency for patients with pain and discomfort.

Research methods
We enrolled IBS patients and collected their clinical data. Patients were classified to the pain only group,
the discomfort only group, and the pain & discomfort group. We compared bowel symptoms, extraintestinal symptoms, IBS-quality of life, psychological status and healthcare-seeking behaviors, and efficacy among the three groups and tested risk factors for symptom reporting in IBS patients.

**Research results**
About one-third of patients meeting Rome III criteria failed to meet Rome IV criteria for an IBS diagnosis. There were no meaningful differences between the pain group and discomfort group for frequency of defecatory abdominal pain or discomfort, bowel habits, coexisting extragastrointestinal pain, comorbid anxiety and depression, and IBS-quality of life scores.

**Research conclusions**
IBS patients with abdominal discomfort have similar bowel symptoms and psychosocial features to those with abdominal pain.

**Research perspectives**
Further studies focused on the pathophysiology and therapeutic response (including the cultural influence) of abdominal pain and discomfort are needed.

**ACKNOWLEDGEMENTS**
The authors thank their colleagues in the Department of Gastroenterology, Peking Union Medical College Hospital for their contributions to the enrollment of IBS patients.

**FOOTNOTES**

**Author contributions:** Fang XC was responsible for study concept and design, data collection and interpretation, and drafting and revision of the manuscript; Fan WJ participated in data collection, data analysis, and figure drafting; Drossman DD was responsible for critical revision; Han SM participated in data analysis; Ke MY participated in critical revision; all authors approved the final version of the manuscript as submitted.

**Supported by** the Program of International S & T Cooperation, No. 2014DFA31850; the National Natural Science Foundation of China, No. 81870379 and No. 81370488; and the Project of the National Key Technologies R & D Program in the 11th Five Year Plan period, No. 2007BAI04B01.

**Institutional review board statement:** This study was reviewed and approved by the Peking Union Medical College Hospital Ethics Committee, No. S-234.

**Informed consent statement:** All study participants provided oral or written consent to participate before study enrollment.

**Conflict-of-interest statement:** There are no conflicts of interest to report.

**Data sharing statement:** No additional data are available.

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

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Randomized Clinical Trial

Peroral endoscopic myotomy vs laparoscopic myotomy and partial fundoplication for esophageal achalasia: A single-center randomized controlled trial

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Abstract

BACKGROUND

Achalasia is a rare benign esophageal motor disorder characterized by incomplete relaxation of the lower esophageal sphincter (LES). The treatment of achalasia is not curative, but rather is aimed at reducing LES pressure. In patients who have failed noninvasive therapy, surgery should be considered. Myotomy with partial fundoplication has been considered the first-line treatment for non-advanced achalasia. Recently, peroral endoscopic myotomy (POEM), a technique that...
employs the principles of submucosal endoscopy to perform the equivalent of a surgical myotomy, has emerged as a promising minimally invasive technique for the management of this condition.

**AIM**
To compare POEM and laparoscopic myotomy and partial fundoplication (LM-PF) regarding their efficacy and outcomes for the treatment of achalasia.

**METHODS**
Forty treatment-naive adult patients who had been diagnosed with achalasia based on clinical and manometric criteria (dysphagia score ≥ II and Eckardt score > 3) were randomized to undergo either LM-PF or POEM. The outcome measures were anesthesia time, procedure time, symptom improvement, reflux esophagitis (as determined with the Gastroesophageal Reflux Disease Questionnaire), barium column height at 1 and 5 min (on a barium esophagogram), pressure at the LES, the occurrence of adverse events (AEs), length of stay (LOS), and quality of life (QoL).

**RESULTS**
There were no statistically significant differences between the LM-PF and POEM groups regarding symptom improvement at 1, 6, and 12 mo of follow-up (P = 0.192, P = 0.242, and P = 0.242, respectively). However, the rates of reflux esophagitis at 1, 6, and 12 mo of follow-up were significantly higher in the POEM group (P = 0.014, P < 0.001, and P = 0.002, respectively). There were also no statistical differences regarding the manometry values, the occurrence of AEs, or LOS. Anesthesia time and procedure time were significantly shorter in the POEM group than in the LM-PF group (185.00 ± 56.89 and 95.70 ± 30.47 min vs 296.75 ± 56.13 and 218.75 ± 50.88 min, respectively; P = 0.001 for both). In the POEM group, there were improvements in all domains of the QoL questionnaire, whereas there were improvements in only three domains in the LM-PF group.

**CONCLUSION**
POEM and LM-PF appear to be equally effective in controlling the symptoms of achalasia, shortening LOS, and minimizing AEs. Nevertheless, POEM has the advantage of improving all domains of QoL, and shortening anesthesia and procedure times but with a significantly higher rate of gastroesophageal reflux.

**Key Words:** Esophageal achalasia; Gastroesophageal reflux; Deglutition disorders; Heller myotomy; Fundoplication; Randomized controlled trial

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**Core Tip:** This randomized controlled trial compared the efficacy and outcomes of laparoscopic myotomy and partial fundoplication (LM-PF) with those of peroral endoscopic myotomy (POEM) for the treatment of patients with achalasia of any etiology. There were no statistically significant differences between the LM-PF and POEM groups regarding symptoms. However, the rates of reflux esophagitis were significantly higher in the POEM group. POEM and LM-PF appear to be equally effective in controlling the symptoms of achalasia, shortening length of hospital stay, and minimizing adverse events. However, POEM has the advantage of shortening anesthesia and procedure times.

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**Citation:** de Moura ETH, Jukemura J, Ribeiro IB, Farias GFA, de Almeida Delgado AA, Coutinho LMA, de Moura DTH, Aissar Sallum RA, Nasi A, Sánchez-Luna SA, Sakai P, de Moura EGH. Peroral endoscopic myotomy vs laparoscopic myotomy and partial fundoplication for esophageal achalasia: A single-center randomized controlled trial. *World J Gastroenterol* 2022; 28(33): 4875-4889

**URL:** [https://www.wjgnet.com/1007-9327/full/v28/i33/4875.htm](https://www.wjgnet.com/1007-9327/full/v28/i33/4875.htm)


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**INTRODUCTION**
Achalasia is a rare benign esophageal motor disorder characterized by incomplete relaxation of the lower esophageal sphincter (LES)[1-3]. For primary or idiopathic achalasia, the underlying etiology has yet to be clearly defined; secondary achalasia results from any one of several systemic diseases including infectious, autoimmune, and drug-induced disorders[4-6]. In both cases, the most common
symptoms are progressive dysphagia, regurgitation, and weight loss. The symptom intensity and treatment response can be assessed with validated scales such as the Eckardt score[2,7,8]. The diagnosis requires the proper integration between reported symptoms and the interpretation of diagnostic tests such as a barium esophagogram, esophagogastroduodenoscopy (EGD), and manometry – either conventional esophageal manometry (EM) or high-resolution manometry (HRM)[9-12].

The treatment of achalasia is not curative but rather is aimed at reducing LES pressure[13-17]. In patients who have failed noninvasive therapy, surgery should be considered[18]. Myotomy with partial fundoplication has been considered the first-line treatment for non-advanced achalasia[19].

Recently, peroral endoscopic myotomy (POEM), a technique that employs the principles of submucosal endoscopy to perform the equivalent of a surgical myotomy, has emerged as a promising minimally invasive technique for the management of this condition[20]. This technique was first described in 1980 and subsequently modified to create what is now POEM[21,22].

This randomized controlled trial (RCT) compared the efficacy and outcomes of laparoscopic myotomy and partial fundoplication (LM-PF) with those of POEM for the treatment of patients with achalasia of any etiology. We also compared the two procedures in terms of the incidence of reflux esophagitis.

MATERIALS AND METHODS

Study design and participants
This was a single-center RCT in which we evaluated 40 treatment-naive patients with esophageal achalasia. We included patients ≥ 18 years of age who had been diagnosed with achalasia based on clinical and manometric criteria (dysphagia score ≥ II and Eckardt score > 3) and who provided informed consent. Patients who had previously undergone endoscopic or surgical procedures involving the esophagogastric junction (EGJ) were excluded, as were those with liver cirrhosis, esophageal varices, Barrett’s esophagus, esophageal strictures, premalignant or malignant EGJ lesions, coagulopathies, pseudoachalasia, esophageal diverticulum, severe cardiopulmonary diseases, or severe systemic diseases, as well as those who were at high surgical risk and those who were pregnant or lactating.

Randomization strategy
An investigator who was unaffiliated with the trial created the randomization list. Specific software (www.randomizer.org) was used, and participants were randomly allocated at a 1:1 ratio to the experimental (POEM) group or the comparison (LM-PF) group.

Sample size calculation
The sample size was calculated to identify statistical significance between LM-PF and POEM regarding reflux esophagitis rates, which were assumed to be 5% and 40% after LM-PF and POEM, respectively [23]. To achieve a power of 80% with an alpha of 0.05, we estimated the minimum sample size to be 38 (19 patients in each group). Taking potential losses into consideration, we chose to include a total of 40 patients.

Techniques
POEM: All POEM procedures were performed by a single operator with extensive experience in the technique. Prophylactic intravenous antibiotics and a proton pump inhibitor (PPI) were administered 30 min before intubation and general anesthesia.

After the gastroscope was introduced, the esophageal lumen and mucosa were thoroughly cleaned. This was followed by submucosal injection of 10 mL of 0.5% indigo carmine. An incision was made into the mucosa of the posterior wall, between 5 and 6 o’clock, at 10 cm above the EGJ. The incision was made with a dual-function submucosal dissection knife (HybridKnife; Erbe, Tübingen, Germany) in Endocut Q mode (effect 2, width 3, and interval 5). Subsequently, spray coagulation (effect 2 at 40 W) was used to create a submucosal tunnel extending 3-4 cm beyond the EGJ into the proximal stomach. In all patients, full-thickness myotomy—including the circular and longitudinal muscle layers—was performed in Endocut Q mode. The myotomy was initiated 2 cm distal from the mucosal entry point and extended 3-4 cm into the proximal stomach. The mucosal incision was closed by using through-the-scope clips (Supplementary Figures 1 and 2).

A barium esophagram was obtained on postoperative day 1. In the absence of complications, the patient was started on a clear liquid diet and subsequently advanced to a full liquid diet for 14 d.

LM-PF: All LM-PF procedures were performed by members of the foregut surgery group. After pneumoperitoneum had been established, five trocars were positioned, after which the left hepatic lobe was retracted to access the EGJ (Supplementary Figure 3). That was followed by division of the phrenoesophageal ligament, dissection, and isolation of the distal esophagus from adjacent structures; and anterolateral dislocation of the distal esophagus. The anterior gastric adipose tissue and the anterior vagus nerve were dissected and separated from the esophagus and stomach, after which myotomy of...
the circular and longitudinal muscle layers was performed, extending from 5-6 cm above the EGJ to 2-3 cm below the EGJ. Partial fundoplication between the esophagus and stomach was then performed by placing sutures in three planes: Posterior—two to three sutures; left lateral—four to five sutures (on the left side); and right anterior—a line of sutures covering the myotomy, thus interposed with the gastric fundus on the right. In the absence of complications, patients were started on a clear liquid diet on the morning following the procedure and maintained on a mechanical soft diet for 14 d after discharge.

**Diagnosis and follow-up**

**Clinical assessments:** Although achalasia subtyping is defined based on HRM, in this study, the achalasia subtype was evaluated according to the degree of esophageal dilatation on the barium esophagogram and esophageal motor activity on EM or HRM. Given that Chagas disease, which often involves the esophagus, is common in Brazil, all patients were screened for anti-*Trypanosoma cruzi* antibodies by enzyme-linked immunosorbent assay and indirect immunofluorescence. Weight loss, dysphagia, and pain were assessed before the procedure, as well as at 6 and 12 mo after the procedure, by using the Eckardt score. Patients with an Eckardt score ≥ 3 were categorized as symptomatic. The clinical evaluation of gastroesophageal reflux (GER) and the diagnosis of GER disease (GERD) was based on the application of the GER Disease Questionnaire (GerdQ) [24] (Supplementary Figure 4).

**EGD:** We performed EGD before the procedure, as well as at 6 and 12 mo after. Esophagitis was graded according to the Los Angeles classification system [25]. We performed chromoendoscopy with narrow-band imaging and 2.5% Lugol’s solution to screen for esophageal cancer. Suspicious lesions were biopsied.

**Barium esophagogram:** To assess esophageal emptying before and 12 mo after the procedure, we used a timed barium esophagogram, as previously described [26]. The degree of esophageal emptying is qualitatively estimated by comparing 1- and 5-min images or by measuring the height and width (in centimeters) of the barium column at both times, calculating the approximate area, and determining the percentage change in the area.

**EM:** Conventional EM was performed before and 12 mo after the procedure. It should be noted that HRM technology was not available in Brazil when the trial began. To perform conventional EM, we used an eight-channel computerized polygraph under pneumohydraulic capillary infusion at a flow rate of 0.6 mL/min/channel. Preparation was required with a 12-h fast and suspension of medications that alter esophageal motility. The technique consists of passing a probe through the nostril and checking the position in the stomach through deep inspiration. With the patient in the supine position, the probe is pulled centimeter by centimeter to measure the mean respiratory pressure and pressure inversion point, and then one of the channels is positioned distal to 3 cm from the upper edge of the LES and the other channels are distant 5 cm apart. Finally, the catheter is pulled up to the upper esophageal sphincter. Through the average of the four distal radial channels of the conventional manometry catheter, the maximum expiratory pressure (MEP) was identified, which best represents the LES pressure itself.

**Quality of life:** To evaluate the quality of life (QoL), we used the Medical Outcomes Study 36-item Short-Form Health Survey (SF-36) [27,28]. The SF-36 comprises 36 questions covering eight domains: Physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health.

**Adverse events:** Among the adverse events (AEs) recorded, pneumoperitoneum requiring drainage or puncture was categorized as a minor AE, as was minor mucosal damage requiring endoscopic closure. Major AEs were defined as pneumoperitoneum leading to hemodynamic instability and premature interruption of the procedure; bleeding requiring a blood transfusion and accompanied by hemodynamic instability or requiring an additional intervention; major mucosal damage requiring endoscopic closure or increasing the length of stay (LOS); or fistula/dehiscence of the incision with signs of fever or infection associated with hemodynamic instability. For AEs occurring in the LM-PF group, we used the Clavien-Dindo classification [29].

**Outcome measures and data collection**

For POEM and LM-PF, the following outcome measures were evaluated: Operative time; length of the myotomy in the esophagus and stomach; myotomy site; complications; and LOS. Patient data were collected on the Research Electronic Data Capture platform.

**Follow-up**

At 1, 6, and 12 mo after the interventions, the Eckardt score was determined, the SF-36 was applied, EGD was performed, and timed barium esophagograms were obtained. Conventional EM was performed at 6 and 12 mo. Patients received the maximum dose of PPI for the first 30 d postprocedure, and those who presented with erosive esophagitis at follow-up endoscopy were maintained on PPI.
treatment for 8 wk. Treatment success was defined as symptom improvement (≤ 3-point reduction in the Eckardt score), an LES pressure < 15 mmHg[30-32], and a > 50% reduction in the height of the barium column at 1 min. Treatment failure was defined as symptom persistence in patients with an Eckardt score ≥ 3.

**Statistical analysis**
We performed descriptive analyses of all study variables. Quantitative variables were expressed as means with standard deviations or as medians with interquartile ranges. Qualitative variables are expressed as absolute and relative frequencies. For the comparison of means between the two groups, the Student’s t-test was used. When the assumption of normality was rejected, the nonparametric Mann-Whitney test was used. To test the homogeneity between proportions, the chi-square test or Fisher’s exact test was used. Repeated-measures analysis of variance was used to compare the groups over the course of the study. When the assumption of normality was rejected, the nonparametric Mann-Whitney and Friedman test were used. The data were processed with the SPSS Statistics software package, version 17.0 for Windows (SPSS Inc., Chicago, IL, United States). P < 0.05 was considered statistically significant.

**RESULTS**

**Population characteristics**
Between March 2017 and February 2018, 40 patients diagnosed with achalasia were enrolled and randomized to undergo LM-PF (n = 20) or POEM (n = 20), as detailed in Figure 1. Nine (22.5%) of the forty patients (five in the POEM group and four in the LM-PF group) tested positive for anti- T. cruzi antibodies, indicating exposure to Chagas disease. At baseline, there was no statistical difference between the two groups in terms of sex, age, etiology, grade, symptom duration, weight, body mass index, or Eckardt score (Table 1). The study was terminated after all patients had been followed for at least 12 mo.

**Treatment outcomes**
Eckardt scores at 1, 6, and 12 mo were lower than the baseline scores in both groups – 1.0, 0.5, and 0.50, respectively, vs 8.0, in the POEM group; and 0.0, 0.0, and 0.0, respectively, vs 8.5, in the LM-PF group—and the differences were statistically significant (P < 0.001; Table 2). There were no statistical differences between the two groups for the Eckardt scores at 1, 6, and 12 mo of follow-up (P = 0.192, P = 0.242, and P = 0.242, respectively).

In the POEM group, treatment success was confirmed at 1 mo in all 20 patients, at 6 mo in 18 of the patients (90%), and at 12 mo in 19 (95%). In the LM-PF group, treatment success was confirmed at 1 mo and was maintained at 6 and 12 mo in all 20 patients. As shown in Table 3, there was no statistical difference between the two groups regarding treatment success at 1, 6, or 12 mo (P = 0.487 and P = 1.000 for 6 and 12 mo, respectively).

In both groups, there were significant postprocedural improvements in dysphagia, although the differences were not significant at 1, 6, or 12 mo (P = 0.602; P = 0.565, and P = 0.547, respectively). However, statistically significant improvements in weight loss, chest pain, and regurgitation were observed in both groups (Supplementary Tables 1 and 2). The postprocedure rate of GER, as assessed with the GerdQ, was higher in the POEM group than in the LM-PF group (64.6% vs 11.1%; P < 0.02).

**Endoscopic findings**
At 1, 6, and 12 mo, only 20, 18, and 18 POEM group patients, respectively, underwent EGD, as did only 17, 16, and 17 LM-PF group patients, respectively. The remaining patients declined to undergo EGD because they were asymptomatic. The rates of esophagitis were significantly higher in the POEM group than in the LM-PF group at 1, 6, and 12 mo of follow-up (P = 0.014, P < 0.001, and P = 0.002, respectively). In the LM-PF group, 1 patient had esophagitis (classified as grade A) at 6 mo and 2 patients had esophagitis (classified as grades B and C, respectively) at 12 mo. In the POEM group, esophagitis was observed at 1 mo in 5 patients (being classified as grade A in one, grade B in three, and grade C in one), at 6 mo in 10 patients (being classified as grade A in three, grade B in two, and grade C in five), and at 12 mo in 11 patients (being classified as grade A in five, grade C in four, and grade D in two). At 1, 6, and 12 mo, the rates of esophagitis were 0.0%, 5.6%, and 11.1%, respectively, in the LM-PF group and 29.4%, 62.5%, and 64.6%, respectively, in the POEM group (Table 4).

**Barium esophagogram**
Table 5 shows the results of the barium esophagogram. In both groups, the heights of the barium column at 1 and 5 min were significantly lower at 1, 6, and 12 mo than at baseline (P < 0.001). There was no statistical difference between the two groups regarding the barium column height values at 1 and 5 min in the follow-up periods (intent-to-treat analysis: P = 0.429 and 0.773; per-protocol analysis: P =
Table 1 Characteristics of the study population

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<td></td>
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<td>LM-PF (n = 20)</td>
<td>POEM (n = 20)</td>
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<td>Male, n (%)</td>
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<td>14 (70.0)</td>
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<td></td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>Chagas disease</td>
<td>9 (22.5)</td>
<td>4 (20.0)</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>31 (77.5)</td>
<td>16 (80.0)</td>
<td>15 (75.0)</td>
</tr>
<tr>
<td>BMI in kg/m², mean ± SD</td>
<td>22.78 (4.49)</td>
<td>22.79 (4.41)</td>
<td>22.77 (4.70)</td>
</tr>
<tr>
<td>Eckardt score, median (IQR)</td>
<td>8.00 (6.25-9.00)</td>
<td>8.50 (7.25-9.75)</td>
<td>8.00 (6.00-9.00)</td>
</tr>
</tbody>
</table>

BMI: Body mass index; IQR: Interquartile range; LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

Table 2 Eckardt scores

<table>
<thead>
<tr>
<th>Time point</th>
<th>Total (n = 40), median (IQR)</th>
<th>Group</th>
<th>P value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM-PF (n = 20), median (IQR)</td>
<td>POEM (n = 20), median (IQR)</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>8.00 (6.25-9.00)</td>
<td>8.00 (6.00-9.00)</td>
<td>0.478</td>
</tr>
<tr>
<td>1 mo</td>
<td>0.00 (0.00-1.00)</td>
<td>0.00 (0.00-1.00)</td>
<td>0.192</td>
</tr>
<tr>
<td>6 mo</td>
<td>0.00 (0.00-1.75)</td>
<td>0.00 (0.00-1.00)</td>
<td>0.242</td>
</tr>
<tr>
<td>12 mo</td>
<td>0.00 (0.00-1.75)</td>
<td>0.00 (0.00-1.00)</td>
<td>0.242</td>
</tr>
</tbody>
</table>

¹Nonparametric Mann-Whitney test.

IQR: Interquartile range; LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

Table 3 Treatment success

<table>
<thead>
<tr>
<th>Time point</th>
<th>Total (n = 40), n (%)</th>
<th>Group</th>
<th>P value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM-PF (n = 20), n (%)</td>
<td>POEM (n = 20), n (%)</td>
<td></td>
</tr>
<tr>
<td>1 mo</td>
<td>40 (100.0)</td>
<td>20 (100.0)</td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>38 (95.0)</td>
<td>20 (100.0)</td>
<td>0.487</td>
</tr>
<tr>
<td>12 mo</td>
<td>39 (97.50)</td>
<td>20 (100.0)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

¹Fisher’s exact test.

LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

0.505 and 0.922).

**EM**

In both groups, the MEP values were significantly lower at 6 and 12 mo than at baseline (Table 6). There was no statistical difference between the two groups at either of those time points (intention-to-treat analysis: P = 0.848).

**AEs, LOS, anesthesia time, and procedure time**

Table 7 describes the AEs, LOS, anesthesia time, and procedure time, in the sample as a whole and by groups. There was no statistical difference between the two groups regarding the rate of AEs (P = 0.605). The relevant complications observed in the immediate postprocedural period included empyema requiring thoracostomy in one (5%) of the LM-PF patients, and inadvertent intraoperative mucosal damage in three (15%) of the POEM patients (treated with endoscopic clipping). The clinical outcomes were favorable in all patients. The mean LOS was 3.95 ± 3.36 d in the LM-PF group, compared with 3.40 ± 0.75 d in the POEM group (P = 0.483). The mean anesthesia time and mean procedure time were both
Table 4 Reflux esophagitis

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>LM-PF, n (%) of Total</th>
<th>POEM, n (%) of Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td>1 (2.5) of 40</td>
<td>0 (0.0) of 20</td>
<td>1.000</td>
</tr>
<tr>
<td>1 mo</td>
<td></td>
<td>5 (13.5) of 37</td>
<td>0 (0.0) of 20</td>
<td>0.014</td>
</tr>
<tr>
<td>6 mo</td>
<td></td>
<td>11 (32.4) of 34</td>
<td>1 (5.6) of 18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>12 mo</td>
<td></td>
<td>13 (37.1) of 35</td>
<td>2 (11.1) of 18</td>
<td>0.002</td>
</tr>
</tbody>
</table>

1Fisher’s exact test.
LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

Table 5 Height of the barium column in cm

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total (n = 40)</th>
<th>Group</th>
<th>LM-PF (n = 20)</th>
<th>POEM (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height at 1 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>17.48 (8.11)</td>
<td>16.97 (6.70)</td>
<td>17.99 (9.46)</td>
<td></td>
</tr>
<tr>
<td>1 mo</td>
<td>9.90 (5.88)</td>
<td>11.39 (4.18)</td>
<td>8.42 (6.99)</td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>8.91 (4.49)</td>
<td>9.61 (3.72)</td>
<td>8.22 (5.15)</td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>10.35 (3.38)</td>
<td>10.98 (2.73)</td>
<td>9.73 (3.90)</td>
<td></td>
</tr>
<tr>
<td>Height at 5 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.31 (8.13)</td>
<td>14.92 (7.06)</td>
<td>15.69 (9.24)</td>
<td></td>
</tr>
<tr>
<td>1 mo</td>
<td>5.53 (5.41)</td>
<td>6.30 (4.84)</td>
<td>4.75 (5.95)</td>
<td></td>
</tr>
<tr>
<td>6 mo</td>
<td>5.69 (5.14)</td>
<td>5.99 (4.56)</td>
<td>5.39 (5.77)</td>
<td></td>
</tr>
<tr>
<td>12 mo</td>
<td>8.31 (4.96)</td>
<td>8.66 (4.92)</td>
<td>7.97 (5.10)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

Table 6 Esophageal manometry results of lower esophageal sphincter pressure in mmHg

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time point</th>
<th>Total (n = 40)</th>
<th>Group</th>
<th>LM-PF (n = 20)</th>
<th>POEM (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal expiratory pressure</td>
<td>Baseline</td>
<td>25.98 (10.42)</td>
<td>24.53 (9.90)</td>
<td>27.43 (10.98)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 mo</td>
<td>10.44 (5.86)</td>
<td>9.93 (5.45)</td>
<td>10.94 (6.34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 mo</td>
<td>10.11 (5.09)</td>
<td>9.34 (4.19)</td>
<td>10.87 (5.85)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

shorter in the POEM group than in the LM-PF group (185.00 ± 56.89 and 95.70 ± 30.47 min, respectively, vs 296.75 ± 56.13 and 218.75 ± 50.88 min, respectively; P < 0.001 for both).

QoL

Table 8 shows the results obtained with the SF-36. In the POEM group, there were postprocedural improvements in all SF-36 domains, whereas there were improvements in only three domains (physical functioning, energy/fatigue, and general health) in the LM-PF group.
Table 7 Adverse events, length of hospital stay, anesthesia time, and procedure time

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total (n = 40)</th>
<th>LM-PF (n = 20)</th>
<th>POEM (n = 20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adverse events, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (10.0)</td>
<td>1 (5.0)</td>
<td>3 (15.0)</td>
<td>0.605c</td>
</tr>
<tr>
<td>Length of hospital stay in d</td>
<td>3.68 (2.42)</td>
<td>3.95 (3.36)</td>
<td>3.40 (0.75)</td>
<td>0.483b</td>
</tr>
<tr>
<td>Anesthesia time in min</td>
<td>240.88 (79.46)</td>
<td>296.75 (56.13)</td>
<td>185.00 (56.89)</td>
<td>&lt; 0.001b</td>
</tr>
<tr>
<td>Procedure time in min</td>
<td>157.23 (74.81)</td>
<td>218.75 (50.88)</td>
<td>95.70 (30.47)</td>
<td>&lt; 0.001b</td>
</tr>
</tbody>
</table>

*a* Fisher’s exact test.  
*b* Student’s *t*-test.  
Data are presented as mean ± SD, unless noted otherwise.  
LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy.

Table 8 Quality of life

<table>
<thead>
<tr>
<th>SF-36 domain</th>
<th>Baseline score</th>
<th>Score at 12 mo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM-PF (n = 20), median (IQR)</td>
<td>POEM (n = 20), median (IQR)</td>
<td>LM-PF (n = 20), median (IQR)</td>
</tr>
<tr>
<td>Physical functioning</td>
<td>95.00 (70.0-100.0)</td>
<td>77.50 (62.25-95.0)</td>
<td>0.165</td>
</tr>
<tr>
<td>Role-physical</td>
<td>100.00 (56.25-100.0)</td>
<td>25.00 (0.00-68.75)</td>
<td>0.012</td>
</tr>
<tr>
<td>Role-emotional</td>
<td>100.00 (33.30-100.0)</td>
<td>33.30 (0.00-66.70)</td>
<td>0.021</td>
</tr>
<tr>
<td>Vitality</td>
<td>67.50 (46.25-88.75)</td>
<td>52.50 (36.25-65.00)</td>
<td>0.052</td>
</tr>
<tr>
<td>Mental health</td>
<td>74.00 (54.0-95.0)</td>
<td>58.00 (44.0-75.0)</td>
<td>0.030</td>
</tr>
<tr>
<td>Social functioning</td>
<td>81.25 (62.50-100.0)</td>
<td>56.25 (37.50-71.88)</td>
<td>0.006</td>
</tr>
<tr>
<td>Bodily pain</td>
<td>73.75 (49.38-97.50)</td>
<td>62.50 (45.0-79.38)</td>
<td>0.201</td>
</tr>
<tr>
<td>General health</td>
<td>50.00 (31.25-83.75)</td>
<td>55.00 (41.25-65.00)</td>
<td>0.698</td>
</tr>
</tbody>
</table>

IQR: Interquartile range; LM-PF: Laparoscopic myotomy and partial fundoplication; POEM: Peroral endoscopic myotomy; SF-36: Medical Outcomes Study 36-item Short-Form Health Survey.

**DISCUSSION**

In this single-center RCT comparing POEM and LM-PF in treatment-naive patients with achalasia, a significant proportion of the patients evaluated had achalasia attributed to Chagas disease. In a study by Farias et al.\[33\], no statistical difference was observed between idiopathic and Chagas disease-associated achalasia regarding treatment success and AEs with POEM.

For years, LM-PF has been considered the gold-standard treatment for achalasia\[34\], because it provides good clinical results, has a low reintervention rate, and has adequate reproducibility. In the first study involving the use of endoscopic myotomy\[21\], conducted in 1980, all 17 of the patients in the sample showed symptom improvement. Although, technical improvements proposed by Inoue et al.\[22\] in 2010 and several cohort studies comparing POEM and LM-PF\[35-45\] over the last decade have proved its safety and efficacy in the management of achalasia, the POEM technique is still not fully standardized\[22\].

The first RCT comparing the two techniques in the treatment of idiopathic achalasia\[46\], including 221 patients, demonstrated clinical success rates at 1 year and 2 years of follow-up of 84.8% and 83.0%, respectively, in the POEM group, comparable to 83.5% and 81.7%, respectively, observed for the LM-PF group. In our study, the clinical success rate at the end of the 1st year was 95% in the POEM group and 100% in the LM-PF group, with no statistical difference between the two techniques. This discrepancy between our results and those of the earlier trial may be related to the fact that approximately 35% of the patients evaluated in that trial had previously received some type of treatment, which could have increased the degree of technical difficulty in dissection secondary to submucosal fibrosis.
We observed no statistical differences between the two techniques concerning Eckardt scores for dysphagia, regurgitation, chest pain, and weight loss, at 1, 6, and 12 mo of follow-up, which demonstrates the noninferiority of POEM to the LM-PF.

Immediate postprocedural complications occurred in 10% of the 40 patients evaluated in the present study. There were no cases of death in our sample, and the rate of AEs did not differ significantly between the two techniques. In our study, all POEM procedures involved a full-thickness myotomy, which made pneumoperitoneum an expected event. Pneumoperitoneum is a common finding after POEM and is not indicative of an unfavorable outcome for the patient. We categorized pneumoperitoneum as an AE only if abdominal decompression was required.

Anesthesia and procedure times were shorter for POEM than for LM-PF. That can be explained by the fact that the POEM involved full-thickness myotomy and did not involve fundoplication. There was no difference between the two procedures in terms of LOS and QoL.

We found that POEM and LM-PF both resulted in significant decreases in the 1- and 5-min barium column heights at 1, 6, and 12 mo after the procedures, demonstrating a clear decrease in resistance to the passage of contrast at the level of the EGJ. Sanagapalli et al. showed an association of significant improvement in symptoms when there is a mean reduction in the residual barium column height by about 53%. The LES pressure (MEP) on conventional EM was significantly lower throughout the follow-up period than at baseline, and there was no significant difference between the two groups.

In this study, the rates of treatment success were comparable between surgical and endoscopic myotomy, both providing symptom improvement, as well as objective improvement in radiological and manometric parameters, at 1, 6, and 12 mo. A recent systematic review and meta-analysis demonstrated that the incidence of GER is higher after POEM than after laparoscopic Heller myotomy. That is in agreement with our findings. The evaluation of GER in our study was based on the typical clinical manifestations of GERD or the identification of erosive esophagitis by EGD. All patients with symptoms and suggestive endoscopic findings of GER received PPI treatment with suspension or maintenance according to the clinical and endoscopic response. A significant limitation of our study was the absence of pHmetry evaluation, which is the main method for GERD evaluation. Prior to our study, we considered that the pHmetry evaluation would be compromised because patients with esophageal achalasia present retention of food residues in the esophageal mucosa and the fermentation of those residues can decrease the intraluminal pH and thus be a confounding factor in the diagnosis of GERD. However, Smart et al. showed that such fermentation would affect only pre-procedure pHmetry,
without much influence on the post-procedure pHmetry.

Erosive esophagitis, especially grade C or D, is considered indicative of GER after endoscopy in patients without a history of the condition [30]. We consider that patients undergoing POEM have a wider esophagogastric transition that favors a higher rate of GER compared to LM-PF, despite similar LES pressures between the groups. Werner et al [46] also showed more GER in patients undergoing POEM despite no differences in manometry compared to LM-PF.

The POEM technique has undergone numerous changes since its initial description by Inoue et al [22]. It has been shown that short- to medium-term efficacy is comparable between myotomy of the circular muscle layer only and full-thickness myotomy, as well as that the latter, despite significantly reducing the duration of POEM, may increase the risk of GER [51,52]. Likewise, there is uncertainty about whether myotomy should be performed in the anterior or posterior wall, the latter technique being associated with a higher incidence of GER [33,54], although other studies have failed to demonstrate that [55,56]. In the present study, we chose a long posterior full-thickness myotomy, because of the greater technical ease [43,57,58].

The results obtained in our study corroborate those of a previous study demonstrating the noninferiority of POEM to LM-PF for symptom control in patients with achalasia, except for postprocedural GER [46]. That raises the question of which technical changes we should study. Therefore, it is valid to perform in-depth analyses of oblique fiber preservation techniques [59], as well as the use of POEM with fundoplication [60,61]. One study [58] demonstrated that preservation of the oblique muscle, using the two penetrating vessels as an anatomical landmark, can significantly reduce the frequency of post-POEM GER, although that should be interpreted with caution because it was a retrospective cohort study, without strict methodological criteria, and with limited reproducibility. In the present study, we employed the conventional POEM technique as previously described [62], and the preservation of the two penetrating vessels was not standardized. The postprocedural occurrence of GERD symptoms in our sample was > 50%, similar to what has been reported by other authors. Despite not including patients undergoing POEM, a recent study [63] showed that achalasia patients with post-treatment reflux symptoms demonstrate esophageal hypersensitivity to chemical and mechanical stimuli, which may determine symptom generation.

Another strategy proposed to minimize the occurrence of GER after POEM is performing transoral incisionless fundoplication. In one pilot study [60], that procedure was reported to have a 100% success rate in terms of symptom control, acid exposure time, and the need for antisecretory drugs. In another pilot study [61], standard POEM combined with endoscopic fundoplication (POEM-F) was employed, and no complications were observed. A recent retrospective study followed patients for 12 mo after POEM-F [64], and showed that the incidence of postprocedural GER was only 11.1%. Albeit attractive, POEM-F has several potential limitations [65]. First, it is necessary to perform POEM in the anterior wall, contrary to the current trend of using a posterior wall approach. Second, it may not be possible to perform POEM-F in patients who have previously undergone anterior myotomy and experience symptom recurrence due to submucosal fibrosis. Third, the long-term durability of this type of fundoplication is still unknown.

In our opinion, it will take some time for the literature to reveal whether endoscopic or surgical myotomy is the best long-term option for the treatment of achalasia. Two crucial points that weigh unfavorably on the POEM procedure, in terms of the possibility that it will come to be widely indicated for the treatment of achalasia [66,67]. The first is the paucity of high-quality (randomized) technical studies comparing POEM with the well-established techniques of pneumatic dilatation of the cardia and laparoscopic myotomy with fundoplication, which could show, at least, the noninferiority of POEM. The second is the lack of studies with long (> 5 years) follow-up periods, which could demonstrate the true reintervention rate, based on the identification of serious late complications, including GER requiring fundoplication and dysphagia resulting from an inadequate myotomy [68]. Currently, the results at 2-3 years are similar between the endoscopic and surgical myotomy techniques concerning the clinical parameters, except for the greater occurrence of GER after the endoscopic technique, which typically responds well to antisecretory drug treatment. However, those data are accompanied by uncertainties that will only be resolved over time.

CONCLUSION

Our results allow us to conclude that LM-PF and POEM are equally effective in controlling the clinical symptoms of achalasia at 1, 6, and 12 mo. Although the use of the POEM technique results in a significantly higher rate of postprocedure GER, it also shortens anesthesia and procedure times. We found no differences between the two methods regarding LOS, the occurrence of AEs, or QoL. In the POEM group, there was an improvement in all domains of QoL.
ARTICLE HIGHLIGHTS

Research background
Achalasia is a rare benign esophageal motor disorder characterized by incomplete relaxation of the lower esophageal sphincter (LES). The treatment of achalasia is not curative but rather aimed at reducing the LES pressure. Surgical myotomy with partial fundoplication is traditional the gold standard method for the management of these patients. Peroral endoscopic myotomy (POEM) uses its increasing due to its satisfactory results.

Research motivation
Since there is still no definition of the best treatment for achalasia, the objective of this study was to compare the techniques used.

Research objectives
This study aimed to compare POEM and laparoscopic myotomy and partial fundoplication (LM-PF) regarding their efficacy and outcomes for the treatment of achalasia.

Research methods
This was a single-center randomized controlled clinical trial.

Research results
There were no significant differences between the LM-PF and POEM groups regarding symptom improvement at 1, 6, and 12 mo of follow-up. Rates of reflux esophagitis were significantly higher in the POEM group. There were also no statistical differences regarding manometry values or the occurrence of adverse events or length of stay. Anesthesia time and procedure time were significantly shorter in the POEM group than in the LM-PF group. In the POEM group, there was an improvement in all domains of quality of life.

Research conclusions
POEM and LM-PF are equally effective in controlling symptoms of achalasia. POEM has the advantage of reducing anesthesia and procedure times, but with a significantly higher rate of gastroesophageal reflux.

Research perspectives
Future research should focus on long-term follow-up and outcomes of different techniques. It is possible that the improvement in the POEM technique may contribute to new perspectives on reflux symptoms.

FOOTNOTES

Author contributions: de Moura ETH contributed to the conception and design of the study; de Moura ETH, Ribeiro IB, de Moura DTH, Aissar Sallum RA, Nasi A, Sánchez-Luna SA, Sakai P, and de Moura EGH analyzed and interpreted the data, drafted the article, revised the article for important intellectual content, and approved the final version; Jukemura J, de Almeida Delgado AA, and Coutinho LMA analyzed and interpreted the data, revised the article for important intellectual content, and approved the final version; Farias GFA analyzed and interpreted the data, and approved the final version.

Institutional review board statement: The study was approved by the Research Ethics Committee of the University of São Paulo School of Medicine, No. CAAE: 2346061300000068.

Clinical trial registration statement: The trial was registered at ClinicalTrials.gov, No. NCT02138643.

Informed consent statement: All subjects agreed to participate in this study after informed consent and ethical permission were obtained.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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Chinese herbal formula shen-ling-bai-zhu-san to treat chronic gastritis: Clinical evidence and potential mechanisms

Wei Jin, Juan Zhong, Yang Song, Ming-Fei Li, Shi-Yi Song, Chun-Run Li, Wei-Wei Hou, Qing-Jie Li

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report’s scientific quality classification
Grade A (Excellent): A, A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gadour E, United Kingdom; Nassar M, United States

Received: January 12, 2022
Peer-review started: January 12, 2022
First decision: March 8, 2022
Revised: March 16, 2022
Accepted: August 6, 2022
Article in press: August 6, 2022
Published online: September 7, 2022

Abstract

BACKGROUND
Chronic gastritis (CG) is an inflammatory disease of the gastric mucosa. Shen-ling-bai-zhu-san (SLBZS), a traditional Chinese medicine formula, is widely used for treating CG. Nevertheless, its effects are currently unclear.

AIM
To determine the clinical evidence and potential mechanisms of SLBZS for the treatment of CG.

METHODS
We systematically searched 3 English (PubMed, Embase, Medline) and 4 Chinese databases (Cochrane Library Central Register of Controlled Trials, China National Knowledge Infrastructure database, Wanfang Data Knowledge Service Platform, and the VIP information resource integration service platform) without language or publication bias restriction. Qualified studies were selected according to pre-set inclusion and exclusion criteria. RevMan 5.3 software was used for meta-analysis and literature quality assessment, Stata 14.0 software was used for sensitivity analysis, GRADE profiler 3.6 was used to evaluate the quality of evidence. And then, network pharmacology analysis was applied to primary research the mechanisms of action of SLBZS on CG.

RESULTS
Fourteen studies were finally included, covering 1335 participants. Meta-analysis indicated that: (1) SLBZS was superior to conventional therapies [risk ratio (RR): 1.29, 95% confidence interval (CI): 1.21 to 1.37, P < 0.00001]; (2) SLBZS was better...
than conventional therapies [RR: 0.24, 95% confidence interval (95%CI): 0.11 to 0.55, \( P = 0.0007 \)] in terms of recurrence rate and reversal of \textit{Helicobacter pylori} positivity (RR: 1.20, 95%CI: 1.11 to 1.30, \( P < 0.00001 \)); and (3) The safety of SLBZS for CG remains unclear. According to the GRADE method, the quality of evidence was not high. Besides, SNZJS might treat CG by acting on related targets and pathways such as EGFR tyrosine kinase inhibitor resistance, the PI3K-Akt signaling pathway, and others.

**CONCLUSION**

SLBZS might be useful in treating CG, but long-term effects and specific clinical mechanisms of it maintain unclear. More samples and high-quality clinical experiments should be assessed and verified in the next step.

**Key Words:** Chronic gastritis; Shen-ling-bai-zhu-san; Chinese herbal formula; Systematic review; Network pharmacology

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Core Tip: A 2012 clinical practice guideline recommended Shenling Baizhu Powder for the Pattern of Spleen and Stomach Deficiency chronic gastritis (CG). The 2020 clinical guideline did not recommended Shen-ling-bai-zhu san (SLBZS), possibly because of inadequate clinical evidence and pharmacological mechanisms. We designed our study to focus on evidence of efficacy and potential mechanisms. Our study showed that SLBZS might be useful in treating CG; however, its long-term effects and mechanisms of action are unclear. Due to the poor quality of the evidence, more samples and high-quality clinical studies should be tested.

Citation: Jin W, Zhong J, Song Y, Li MF, Song SY, Li CR, Hou WW, Li QJ. Chinese herbal formula shen-ling-bai-zhu-san to treat chronic gastritis: Clinical evidence and potential mechanisms. \textit{World J Gastroenterol} 2022; 28(33): 4890-4908
URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4890.htm
DOI: https://dx.doi.org/10.3748/wjg.v28.i33.4890

**INTRODUCTION**

Chronic gastritis (CG) is a set of inflammatory diseases of the gastric mucosa[1] and is one of the common diseases of the digestive system. The disease often relapses, accompanied by symptoms that severely affect the quality of life. Chronic atrophic gastritis is associated with intestinal metaplasia and intraepithelial neoplasia, increasing gastric cancer risk. Globally, on average, more than 50% of people may have CG at any given moment[2]. A pathological study of 8892 patients in China found that atrophic gastritis, intestinal metaplasia, and dysplasia were prevalent, occurring in 25.8%, 23.6%, and 7.3% of the population, respectively[3].

The treatment of CG with gastric mucosal repair consists of antacids, antacids, and gastric mucosal protective agents[4–7]. Nevertheless, the efficacy of triple or quadruple therapy is not ideal, and there are frequent side effects[8]. For these reasons, complementary and alternative medicine therapies such as acupuncture[9–12], moxibustion[13,14], and Chinese herbal formulas[15,16] are sought as alternative therapies.

The Chinese herb formula Shenling Baizhu Powder, also known as Shen-ling-bai-zhu-san (SLBZS), is a widely used prescription for digestive tract disease in China derived from the classic herb monograph “\textit{Taipingguaminhejijufang}” written in the Song dynasty[17]. Ten commonly used herbs constitute SLBZS; these include Baizhu (\textit{Atractylodes macrocephala} Koidz), Fuling (\textit{Smilax glabra} Roxb), Yiyiren (\textit{Coix lacryma-jobi} var. \textit{ma-yuen} (Rom.Caill.) Stapf), Renshen (\textit{Panax ginseng} C.A.Mey), Shanyao (\textit{Dioscorea oppositifolia} L), Baibiandou (\textit{Lablab purpureus} subsp. \textit{purpureus}), Lianzi (\textit{Nelumbo nucifera} Gaertn), Sharen (\textit{Amomum villosum} Lour), Jiegeng (\textit{Platycodon grandiflorus}) and Gancao (\textit{Glycyrrhiza uralensis} Fisch. ex DC). In China, clinical studies suggested that SLBZS treats CG[18,19] with efficacy. Nevertheless, mechanistic studies based on animal experiments are lacking.

Furthermore, a 2012 Clinical practice guideline[20] recommended Shenling Baizhu Powder for the Pattern of Spleen and Stomach Deficiency CG. The pathogenesis can be summarized as the stomach failing to be nourished because of splenic and gastric qi deficiency and disturbance of qi movement. A 2020 clinical guideline did not recommend SLBZS, possibly because of inadequate clinical evidence and pharmacological mechanisms[21]. Efficacy evidence and potential mechanistic studies are required.
### Table 1 Search strategy

<table>
<thead>
<tr>
<th>Number</th>
<th>Search terms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mesh descriptor (Medicine, Traditional) explode all trees</td>
</tr>
<tr>
<td>2</td>
<td>(Medicine, Chinese Traditional*): ti,ab,kw</td>
</tr>
<tr>
<td>3</td>
<td>Mesh descriptor(Drugs, Chinese Herbal) explode all trees,</td>
</tr>
<tr>
<td>4</td>
<td>((Chinese Drugs, Plant*) or (Chinese Herbal Drugs*) or (Herbal Drugs, Chinese*) or (Plant Extracts, Chinese*) or (Chinese Plant Extracts*) or(Extracts, Chinese Plant*)): ti,ab,kw</td>
</tr>
<tr>
<td>5</td>
<td>Mesh descriptor (shen-ling-bai-zhu) explode all trees</td>
</tr>
<tr>
<td>6</td>
<td>((shen-ling-bai-zhu powder*) or (shen-ling-bai-zhu formula*) or (shen-ling-bai-zhu decoction*) or (shen-ling-bai-zhu decoction*) or (Shen-ling-bai-zhu powder*) or (Shen-ling-bai-zhu formula*) or (Shen-ling-bai-zhu formula*)): ti,ab,kw</td>
</tr>
<tr>
<td>7</td>
<td>Or 1-6</td>
</tr>
<tr>
<td>8</td>
<td>Mesh descriptor: (Chronic gastritis) explode all trees</td>
</tr>
<tr>
<td>9</td>
<td>((Chronic gastritis*) or (Digestive System Diseases*) or (Gastrointestinal Diseases*) or (Gastroenteritis*) or (Gastritis*) or (Chronic, gastritis*)): ti, ab, kw</td>
</tr>
<tr>
<td>10</td>
<td>Or 8-9</td>
</tr>
<tr>
<td>11</td>
<td>Mesh descriptor: (randomized controlled trials) explode all trees</td>
</tr>
<tr>
<td>12</td>
<td>(random* or (randomly*) or (allocation*) or (random allocation*) or (placebo* or (double blind*) or (clinical trials*) or (randomized control trial*) or (RCT*) or (controlled clinical trials*): ti, ab, kw</td>
</tr>
<tr>
<td>13</td>
<td>Or: 11-12</td>
</tr>
<tr>
<td>14</td>
<td>7 and 10 and 13</td>
</tr>
</tbody>
</table>

### MATERIALS AND METHODS

#### Study registration

We registered this review and meta-analysis at the PROSPERO website ([https://www.crd.york.ac.uk/PROSPERO/#recordDetails](https://www.crd.york.ac.uk/PROSPERO/#recordDetails)), an international prospective system review registration website. The registration number was CRD42020212979. We conducted the study based on the details of this protocol.

#### Database search

Our investigators independently searched PubMed, Embase, Medline, Cochrane Library Central Register of Controlled Trials, China National Knowledge Infrastructure database, Wanfang Data Knowledge Service Platform, and the VIP information resource integration service platform from their inception to November 2021. There were no limitations on language or publication status. They also searched conference articles and clinical registries for possible related trials.

#### Search terms

We adopted a search strategy that combined medical subject headings and free words. Two authors (YS, MFL) searched and screened all citations independently. The search strategy was as follows (Table 1): The search strategy followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[22].

#### Inclusion criteria

Randomized controlled trials (RCTs) or quasi-RCTs that reported the effects of SLBZS on CG were included.

Participants: Studies that evaluated patients with a diagnosis of CG were included. For example, we used diagnostic criteria from the standardized consensus on the diagnosis of CG from the Branch of Spleen and Stomach Diseases of the Chinese Society of Traditional Chinese Medicine, China, that depends on endoscopy and pathological examinations[23]. We excluded studies that included CG patients complicated with hypertension, diabetes, heart disease, or severe allergic diseases. There was no restriction on the setting of interest or other population characteristics.

Interventions: SLBZ powder was the primary prescription, regardless of its dosage form, dosage, or course of treatment. If there were other medications, formulas, or traditional Chinese medicine (TCM) therapies (such as acupuncture, moxibustion, and ear-acupressure) in the treatment group, the control groups must also receive these therapies.
Comparisons: Western medicine, active control, and placebo were acceptable. If SLBZ + western medicine was applied in the experimental group, western medicine in the control group must be consistent.

Outcome measures: We considered efficacy outcomes as primary outcome measures, including effectiveness, recurrence rate, symptom score, Helicobacter pylori (H. pylori) eradication, and quality-of-life assessment. Secondary outcome measures were adverse events directly related to CG.

Exclusion criteria
The exclusion criteria were as follows: (1) The study was not an RCT, e.g., retrospective study, cross-sectional study, observational study, case study, animal study, or others; (2) for multiple reports or repeated publications from the same study, we retained the one with a more significant number of details; (3) diagnostic criteria were not reported in trials, disease not CG; and (4) studies or trials used SLBZS as a part of complex interventions; for example, SLBZS decoction plus another herbal medicine formula vs acupuncture therapies. Western medicine is inconsistent in two groups.

Study selection and data extraction
According to our study registration protocol, two reviewers (WJ, QJL) independently performed trial searches, study selection, and raw data extraction. A third reviewer (JZ) checked the extracted data. We resolved conflicts through consensus.

Risk of bias assessment
According to the Cochrane Handbook details[24], we performed the risk of bias assessment analysis using the Cochrane collaborative bias risk tool in Review Manager 5.3 software. We resolved conflicts by consultation with a third investigator (WWH).

Statistical analysis
We used Review Manager 5.3 and Stata 14.0 software for statistical analysis. We calculated 95% confidence interval (CI) and mean difference for continuous variables and 95%CI and risk ratio (RR) for dichotomous variables. Differences with \( P < 0.05 \) were statistically significant. We determined the heterogeneity of data using Cochrane \( \chi^2 \) and \( P \) tests. We used a fixed-effect model if there was no significant heterogeneity; otherwise, we used a random-effect model. We conducted subgroup analyses to explore the source of heterogeneity. We determined publication bias by examining funnel plots and Egger’s tests for more than ten trials. We used sensitivity analysis to explore the stability of the results. GRADE profiler 3.6 software was applied to evaluate the quality of evidence.

Mechanisms of network pharmacology of SLBZD to treat CG
Collection and screening of pharmacodynamic components in TCM System Pharmacology Database and analysis platform (TCMSP, HTTP://ibts.hkbu.edu.hk/LSP/tcmsp.php) in ginseng, atractyloides, poria cocos, yam, white hyacinth bean, lotus seed, coix seed, amomum fruit, radix platycodi, radix glycyrrhizae as keyword query filter chemical composition. The database contains about 500 drugs listed in the Chinese Pharmacopoeia, providing absorption, distribution, metabolism and excretion, ingredient data, and target and disease information. Oral bioavailability (OB) and drug-like properties (DL) are essential indexes determining whether a compound can be developed into a drug. Based on the relevant literature, OB and DL were set to > 30% and > 0.18, respectively, and the screened compounds were used as candidate ingredients[25,26].

Target prediction of pharmacodynamic components, the simplified molecular Linear Input specification (Simles) number, and Mol structure of each candidate component were retrieved using PubChem. We arranged candidate target genes using PharmMapper online (http://Lilab-ecust.cn/pharmmapper/index.html) and Swiss target prediction (HTTP://www.swisstargetprediction.ch/), and we arranged the standbys in an Excel form.

Prediction of disease Targets Genes associated with CG was identified by searching for “Chronic Gastritis” in GeneCards (http://www.genecards.org/).

Network construction and analysis
SLBZ Powder’s candidate components and target genes were screened and imported into Cytoscape 3.7.2 software using Excel to obtain a component-target network diagram. The predicted disease candidate targets were imported into the online protein interaction (String) database, the species organism was set as human (Homo sapiens), and the PPI map was obtained. The PPI map was imported into Cytoscape 3.7.2 software. The potential targets of Shenlingbaizhu Powder in chronic gastritis can be obtained by merging the component-target network diagram and disease target PPI diagram using Merge software, which can be imported into the online String database the interaction map of potential targets.

Functional mechanism analysis of potential targets GO enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment annotation analysis of potential target genes were performed using the R package clusterProfiler.
Table 2 Excluded 11 studies after reading the full text

<table>
<thead>
<tr>
<th>Reason</th>
<th>Ref. (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervention combined with other decoction (n = 3)</td>
<td>Li GS. Observation on the curative effect of Shenling Baizhu Powder and Taohong Siwu Decoction in treating chronic gastritis. Zhongguo Linchuang Zazhi 2007; 19 (10): 260-261</td>
</tr>
<tr>
<td>Intervention combined with other decoction, no diagnostic criteria (n = 2)</td>
<td>Jin JZ. Shenling Baizhu Powder and Zuoqin pill to treat chronic gastritis. Shiying Zhongyi Neike Zazhi 2011; 27 (11): 752</td>
</tr>
<tr>
<td></td>
<td>Gao CZ. Yang SM. Observation on curative effect of cefaclor combined with Shenlingbaizhu granule and Muxiang Shunqi pill in treating chronic gastritis. Zhongjia Yixue Chuanxiangxin Zazhi 2012; 9 (22): 127-128</td>
</tr>
<tr>
<td>No diagnostic criteria (n = 1)</td>
<td>Shi ZR. Clinical observation on 8 cases of chronic gastritis treated by Shenling Baizhu Powder. Neimenggu Zhongyi Zazhi 2014 [DOI: 10.16040/j.cnki.cn15-1101.2014.07.024]</td>
</tr>
<tr>
<td>Not CG(n = 1)</td>
<td>Zhang WW. Clinical observation on 96 cases of spleen deficiency and stomachache treated with Shenling Baizhu Powder. Zhongguo Minzujiexue Ya Minzujuanjue 2013; 9 (12): 80</td>
</tr>
<tr>
<td>Intervention combined with acupuncture (n = 3)</td>
<td>Yang FX. Acupuncture combined with Shenling Baizhu Powder to treat chronic gastritis with spleen deficiency and dampness. Kouqiang Yixue Dianzi Zazhi 2015; 6 (13): 140-143</td>
</tr>
<tr>
<td></td>
<td>Wu XR. 30 cases of chronic gastritis with spleen deficiency and dampness treated by acupuncture combined with Shenling Baizhu Powder. Guangming Zhongyi 2015; 30 (5): 1018-1020</td>
</tr>
<tr>
<td>Intervention combined with other decoction, WM are inconsistent in two groups (n = 1)</td>
<td>Yan Z. Clinical study of cefaclor combined with Shenling Baizhu granule and Muxiang Shunqi pill in treating chronic gastritis. Yatui Chuantong Yiyue 2015; 31 (18): 106-107</td>
</tr>
</tbody>
</table>

RESULTS

Database search results
We retrieved 335 trials from 6 databases. When duplicate records were deleted, 177 remained. We excluded 152 studies by reading the title and abstract of the papers, including seven repeatedly published studies, ninety-five reports on the SLBZS experience of experienced TCM doctors, four retrospective studies, seventeen observation studies, one case report, and twenty-eight studies of diseases that were not CG. We read the full texts of the remaining 25 records. We deleted 11 records because of exclusion criteria (Table 2).

Finally, we included 14 studies in our review (flowchart of database search and study identification is shown in Figure 1).

Study characteristics
There were 14 Chinese-language RCTs, comprising 1335 participants aged 15-68 years[27-40], published between 2008 and 2020. Interventions in these studies were SLBZS vs conventional medicine or SLBZS + conventional medicine vs conventional medicine. In conventional medicine therapy, there were four methods, including monotherapy in four trials[27,33,34,37], combined therapy in one study[36], triple therapy in seven studies[28,31,32,35,38,40] and two trials of quadruple therapy[29,30]. There were various treatment durations, including 4, 5, 8, and 12 wk.

Total effectiveness was the primary outcome measure in all trials. All trials reported balanced baseline characteristics. Five trials (36%) recorded adverse events[29,31,34,36,38], and three studies reported recurrence rates[34,38,40]. Two studies reported participant withdrawal information[31,34]. No study reported influence on the quality of life as an outcome measure. Characteristics of included studies are shown in Table 3.

Risk of bias assessment
(1) Fourteen trials were consistent at baseline, and all tests referred to RCTs, three studies[28,39,40] mentioned randomization using the “random number table” method; (2) all studies not reported “distribution hidden” method; (3) the “blinding method” was not reported in any study, two studies[31,34] reported “No cases withdrawal and dropped-out,” and three studies[34,38,40] reported “recurrence rate”; (4) selective reporting may come out in studies that there were too few indicators were noted; and (5) we considered some support from pharmaceutical companies that the ethics committee would not approve as other bias. If herbs were offered free by pharmaceutical companies, bias might taint the results. Two studies[34,36] reported that an ethics committee approved the study, suggesting a low bias level. For another 12 studies, we could not determine the effects of other potential sources of bias.
Table 3 Characteristics of included studies

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study design</th>
<th>Sample size (E/C)</th>
<th>Gender (E/C) and age (yr)</th>
<th>Duration</th>
<th>Interventions</th>
<th>Control group</th>
<th>Experimental group</th>
<th>Period</th>
<th>Outcome measure</th>
<th>Balance report of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yu et al. [27], 2014</td>
<td>RCT</td>
<td>48 (24/24)</td>
<td>(13/11); (10/14); (34.96 ± 11.39)/ (34.08 ± 12.82)</td>
<td>Not mentioned</td>
<td>Rabeprazole enteric-coated capsule</td>
<td></td>
<td>Rabeprazole enteric-coated capsule + SLBZD</td>
<td>4 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Chen et al. [28], 2014</td>
<td>RCT</td>
<td>79 (40/39)</td>
<td>(24/16); (25/16); (42.6 ± 13.1)/43.5 ± 13.4</td>
<td>6-17 mo/6-19 mo</td>
<td>Triple therapy (clarithromycin sustained-release tablets + rabeprazole sodium capsule + metronidazole tablets)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>4 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Chen et al. [29], 2018</td>
<td>RCT</td>
<td>60 (30/30)</td>
<td>(15/16); (25/16); (55.45 ± 6.55)/ (55.46 ± 6.44)</td>
<td>3-12 mo</td>
<td>Quadruple therapy (rabeprazole sodium capsule + amoxicillin + clarithromycin sustained-release tablets + biskal-citrate)</td>
<td></td>
<td>Quadruple therapy + SLBZD</td>
<td>8 wk</td>
<td>Effective rate; H. Pylori eradication; adverse event</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Du [30], 2017</td>
<td>RCT</td>
<td>48 (26/22)</td>
<td>(14/12); (12/10); (40.7 ± 6.1)/ (41.2 ± 6.6)</td>
<td>7 mo-9 years/6 mo-8 years</td>
<td>Quadruple therapy (amoxicillin clavulanic potassium chewable tablets + metronidazole + omeprazole + compound bismuth aluminale capsule)</td>
<td></td>
<td>SLBZD</td>
<td>5 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Ga [31], 2017</td>
<td>RCT</td>
<td>98 (49/49)</td>
<td>Not mentioned; 19-58</td>
<td>Not mentioned</td>
<td>Triple therapy (omeprazole + clarithromycin + amoxicillin)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>4 wk</td>
<td>Effective rate; H. Pylori eradication rate; adverse event</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Li et al. [32], 2020</td>
<td>RCT</td>
<td>66 (33/33)</td>
<td>(19/14); (18/15); (58.54 ± 4.65)/ (58.62 ± 4.57)</td>
<td>4-17 years/4-18 years</td>
<td>Triple therapy (mosapride tablet + polyzyme tablets + lansoprazole tablets)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>12 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Tang [33], 2014</td>
<td>RCT</td>
<td>60 (30/30)</td>
<td>(16/14); (17/15); (22-46)/ (23-52)</td>
<td>Not mentioned</td>
<td>Omeprazole enteric-coated capsules</td>
<td></td>
<td>Omeprazole Enteric-coated Capsules + SLBZD</td>
<td>8 wk</td>
<td>Effective rate; recurrence rate; adverse event</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Xia [34], 2015</td>
<td>RCT</td>
<td>300 (150/150)</td>
<td>Not mentioned; 18-85</td>
<td>Not mentioned</td>
<td>Omeprazole enteric-coated capsules</td>
<td></td>
<td>SLBZD</td>
<td>8 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Xu et al. [35], 2018</td>
<td>RCT</td>
<td>60 (30/30)</td>
<td>(17/13); (16/14); (55.6 ± 16.4)/ (56.8 ± 14.9)</td>
<td>4-20 years/4-19 years</td>
<td>Triple therapy (mosapride tablet + polyzyme tablets + lansoprazole tablets)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>12 wk</td>
<td>Effective rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Zhang et al. [36], 2020</td>
<td>RCT</td>
<td>68 (34/34)</td>
<td>(15/19); (17/17); (44.8 ± 5.0)/ (45.2 ± 5.4)</td>
<td>1-12 years/2-14 years</td>
<td>Combination therapy (omeprazole + compound bismuth aluminale granules)</td>
<td></td>
<td>Combination therapy + SLBZD</td>
<td>8 wk</td>
<td>Effective rate; adverse events</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Zhao and Lin [37], 2010</td>
<td>RCT</td>
<td>80 (40/40)</td>
<td>(37/3); (38/2); (46.2 ± 6.7)/ (44.2 ± 5.7)</td>
<td>2-7 years/2-8 years</td>
<td>No alcohol, famotidine</td>
<td></td>
<td>No alcohol, famotidine + SLBZD</td>
<td>4 wk</td>
<td>Effective rate;</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Zheng [38], 2014</td>
<td>RCT</td>
<td>92 (46/46)</td>
<td>(28/18); (30/16); (34 ± 5.5)/ (33 ± 5.76)</td>
<td>5 mo-6 years/7 mo-6 years</td>
<td>Triple therapy (amoxicillin dispersion tablet + omeprazole enteric-coated capsul + clarithromycin tablet)</td>
<td></td>
<td>SLBZD</td>
<td>4 wk</td>
<td>Effective rate; adverse events; recurrence rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Zhuang et al. [39], 2019</td>
<td>RCT</td>
<td>106 (53/53)</td>
<td>(65/41); (46.20 ± 8.75)</td>
<td>1-11 years</td>
<td>Triple therapy (omeprazole- enteric-coated tablets + clarithromycin dispersible tablets + amoxil capsule)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>4 wk</td>
<td>Effective rate; H. Pylori’s negative conversion rate</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Zou [40], 2015</td>
<td>RCT</td>
<td>170 (85/85)</td>
<td>(86/84); (40.9 ± 11.1)</td>
<td>Not mentioned</td>
<td>Triple therapy (amoxicillin + clarithromycin + omeprazole)</td>
<td></td>
<td>Triple therapy + SLBZD</td>
<td>8 wk</td>
<td>Effective rate; H. Pylori’s negative</td>
<td>$P &gt; 0.05$</td>
</tr>
</tbody>
</table>
RCT: Randomized controlled trial; *H. Pylori*: *Helicobacter pylori*; SLBZS: Shen-ling-bai-zhu san.

**Evaluation of outcome measures**

**Total effectiveness:** Total effectiveness is a composite endpoint composed of improved symptoms and
Table 4 Methodological quality details of all included studies

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Baseline</th>
<th>Randomization</th>
<th>Allocation concealment</th>
<th>Blind method</th>
<th>Withdrawal or dropped-out</th>
<th>Follow up</th>
<th>Protocol and registration</th>
<th>Ethics committee approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yun[27], 2014</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Chen et al[28], 2014</td>
<td>Comparability</td>
<td>Random number</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
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</tr>
<tr>
<td>Chen et al[29], 2018</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
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</tr>
<tr>
<td>Du[30], 2017</td>
<td>Comparability</td>
<td>Random</td>
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<td>NR</td>
<td>NR</td>
<td>NR</td>
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<td>NR</td>
</tr>
<tr>
<td>Gu[31], 2017</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>No cases withdrawal and dropped-out</td>
<td>NR</td>
<td>NR</td>
<td>Approved</td>
</tr>
<tr>
<td>Li et al[32], 2020</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Approved</td>
</tr>
<tr>
<td>Tang[33], 2014</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Xia[34], 2015</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>No cases withdrawal and dropped-out</td>
<td>Recurrence rate</td>
<td>NR</td>
<td>Approved</td>
</tr>
<tr>
<td>Xu et al[35], 2018</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zhang et al[36], 2020</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zhao and Lin[37], 2010</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zheng[38], 2014</td>
<td>Comparability</td>
<td>Random</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Recurrence rate</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zhuang et al[39], 2019</td>
<td>Comparability</td>
<td>Random number</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zou[40], 2015</td>
<td>Comparability</td>
<td>Random number</td>
<td>NR</td>
<td>NR</td>
<td>Recurrence rate</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR: Not Reported.

gastroscopy. The results fall into three categories: Obviously effective, effective, and invalid, according to clinical Research on New Chinese Medicines[41]. The details are as follows. Clinical cure: Epigastric pain and symptoms disappeared, gastroscopy returned to normal, i.e., gastric mucosa repair, the disappearance of active inflammation, and mild chronic inflammation; Obviously effective: Epigastric pain and symptoms disappear or diminish. Gastroscopy showed significant improvement; that is, gastric mucosa was nearly normal, active inflammation was gone, and there was less chronic inflammation; Effective: Relief of epigastric pain and other symptoms. Gastroscopy showed reduced gastric mucosal lesions; that is, gastric mucosa was essentially normal, active inflammation was gone, and less chronic inflammation; and Invalid: no improvement or aggravation of clinical symptoms and signs. Gastroscopy showed no change. There were slight differences in this outcome’s composition in various studies due to the non-uniform efficacy assessment criteria. All 14 RCTs compared the total effectiveness rate of SLBZS in patients with CG. SLBZS was superior to conventional therapies (RR: 1.29, 95% CI: 1.22 to 1.37, \( P < 0.00001 \)) (Figure 3A). Heterogeneity in the total effectiveness was very small (\( P = 0.91, I^2 = 0\% \)).

We created subgroups based on the duration of treatment (4, 5, 8, or 12 wk) (Supplementary Table 1), comparison type (SLBZS vs conventional medicine or SLBZS + conventional medicine vs conventional medicine alone) (Supplementary Table 2), and intervention method (monotherapy, combined therapy, triple therapy, or quadruple therapy) (Supplementary Table 3). These subgroup analyses showed that the effectiveness rate of SLBZS did not differ based on the duration of treatment, combination with other medications, or intervention method (all \( P > 0.05 \)) (Table 5).
Table 5 Subgroup analysis of total effectiveness

<table>
<thead>
<tr>
<th>Subgroup method (total effective rate)</th>
<th>Items</th>
<th>Number of comparisons</th>
<th>Results (risk ratio, 95%CI)</th>
<th>P value for overall effect</th>
<th>I²</th>
<th>P value for subgroup difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Course of treatment</td>
<td>All comparisons</td>
<td>14</td>
<td>1.29 (1.22, 1.37)</td>
<td>&lt; 0.00001</td>
<td>0%</td>
<td>NA</td>
</tr>
<tr>
<td>Supplementary Table 1</td>
<td>4 wk</td>
<td>5</td>
<td>1.27 (1.17, 1.37)</td>
<td>&lt; 0.00001</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>5 wk</td>
<td>1</td>
<td>1.45 (1.04, 2.03)</td>
<td>0.03</td>
<td></td>
<td>NA 0.58</td>
</tr>
<tr>
<td></td>
<td>8 wk</td>
<td>5</td>
<td>1.28 (1.16, 1.40)</td>
<td>0.02</td>
<td></td>
<td>0%</td>
</tr>
</tbody>
</table>
|                                     | 12 wk                      | 2                     | 1.44 (1.19, 1.74)           | 0.0002                    |    | 0%                             |<|-
| Comparison type                     | All comparisons            | 14                    | 1.23 (1.14, 1.32)           | < 0.00001                 | 47%|                                |
| Supplementary Table 2               | SLBZS vs CM                | 3                     | 1.23 (1.10, 1.38)           | 0.0003                    |    | 0% 0.93                       |
|                                     | SLBZS + CM vs CM           | 11                    | 1.25 (1.11, 1.35)           | < 0.0001                  | 57%|                                |
| Intervention method                 | All comparisons            | 14                    | 1.29 (1.22, 1.37)           | < 0.0001                  | 0% |                                |
| Supplementary Table 3               | Monotherapy                | 4                     | 1.25 (1.12, 1.40)           | < 0.0001                  |    | 5%                             |
|                                     | Combined therapy           | 1                     | 1.41 (1.08, 1.84)           | 0.01                      |    | NA 0.82                       |
|                                     | Triple therapy             | 7                     | 1.30 (1.21, 1.40)           | < 0.0001                  |    | 0%                             |
|                                     | Quadruple therapy          | 2                     | 1.35 (1.11, 1.64)           | 0.003                     |    | 0%                             |

NA: Not available; CM: Conventional medicine; SLBZS: Shen-ling-bai-zhu san.

Table 6 Adverse events

<table>
<thead>
<tr>
<th>Study</th>
<th>Experiment group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhang, 2020</td>
<td>Diarrhea (2/34)</td>
<td>Dizziness (2/34) and dry mouth (1/34)</td>
</tr>
<tr>
<td>Chen, 2018</td>
<td>Headache (1/30), diarrhea (1/30), nausea (1/30)</td>
<td>Headache (2/30), diarrhea (1/30), nausea (2/30), constipation (1/30), rash (1/30)</td>
</tr>
<tr>
<td>Zheng, 2014</td>
<td>None</td>
<td>Headache and rash (17.39%)</td>
</tr>
</tbody>
</table>

Recurrence rate: Three studies reported recurrence rate[34,38,40]. Pooled raw data showed that SLBZS was better than conventional therapies (RR: 0.24, 95%CI: 0.11 to 0.55, P = 0.0007, Figure 3B).

HP negative conversion rate: Four trials noted the reversal rate for *Helicobacter pylori* (*H. pylori*) positivity[29,31,39,40]. Meta-analysis showed that SLBZS was superior to conventional therapies (RR: 1.20, 95%CI: 1.11 to 1.30, P < 0.00001, Figure 3C).

Other results

One trial compared the time required for symptom improvement in patients with CG[38]. The experimental group was superior to the control group regarding effects on epigastric stagnation, abdominal distension, belching, acid regurgitation, and nausea (P < 0.05).

There were no reports of significant responses or improvement in the quality-of-life data in these studies. One study reported the Questionnaire for Comprehensive Quality of Life Assessment responses pre- and post-treatment in two groups[36]. After two consecutive months of treatment, scores in all dimensions improved, and the treatment group’s score was significantly higher than that of the treatment group (P < 0.05).

Publication bias

Funnel plots showed the publication bias of the effectiveness rate (Figure 4A). The funnel plot of the effective rate was symmetric, suggesting no significant publication bias. Egger’s test results agreed with the funnel plots (P = 0.005 and 0.000, respectively).

Adverse events

Of the 14 studies, nine RCTs did not mention adverse events[27,28,30,32,33,35,36,39,40]. Two studies
Table 7 GRADE evidence for the effect of Shen-ling-bai-zhu san

<table>
<thead>
<tr>
<th>Quality assessment</th>
<th>No of patients</th>
<th>Effect</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of studies</td>
<td>Design</td>
<td>Limitations</td>
</tr>
<tr>
<td>Effective rate</td>
<td>14</td>
<td>Randomized trials</td>
<td>Serious¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrence rate</td>
<td>3</td>
<td>Randomized trials</td>
<td>Serious¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP negative conversion rate</td>
<td>4</td>
<td>Randomized trials</td>
<td>No serious limitations¹</td>
</tr>
</tbody>
</table>

¹Randomized controlled trial design method is not reported.
²The risk of bias assessment is mostly “unclear risk” because there are not enough details in articles.
³Studies come from China.
⁴There is significant heterogeneity between studies.

mentioned no prominent adverse events[^34,29]. Three trials reported adverse events (Table 6); however, no study commented on methods used to manage these events.

**GRADE evidence for the effect of SLBZD**

GRADE results of SLBZD is shown in (Table 7). However, the quality of evidence was very low or moderate because of the poor methodological quality.

**Network pharmacology results of SLBZS**

**Composition and targets of SCBZS:** According to the OB > 30% and DL > 0.18 standard screening, we screened 189 ingredients, including seven in Baizhu (Atractylodes macrocephala Koidz), 15 in Fuling (Smilax glabra Roxb), 9 in Yiyiren [Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf], 22 in Renshen (Panax ginseng C.A.Mey), 15 in Shanyao (Dioscorea oppositifolia L), one in Baibiandou (Lablab purpureus subsp. purpureus), 11 in Lianzi (Nelumbo nucifera Gaertn.), 92 in Gancao (Glycyrrhiza uralensis Fisch. ex
DC), ten in Sharen (*Amomum villosum* Lour.), and seven in Jiegeng (*Platycodon grandiflorus*). The repeated components and components with no target were deleted, leaving 158 candidate components. Each candidate component’s top 15 target genes were selected, and duplicated genes were identified, with 693 candidate target genes.

**PPI network:** The component-target network diagram of Shen-ling-bai-zhu Powder visually shows the interaction between pharmacodynamic components and target genes of Shen-ling-bai-zhu Powder (Figure 4B). The network contains 851 nodes with 2445 sides, among which 158 nodes represent candidate ingredients and 693 nodes represent candidate target genes related to drug candidate ingredients. The average number of neighborhood nodes was 5.561. There were 300 nodes and 3325 edges in the disease target interaction network, and the average number of neighborhood nodes in the network was 34.635. A total of 35 potential targets of SLBZS on chronic gastritis can be obtained by analyzing the component-target and disease target interaction networks. Figure 4C visually shows the interaction relationship between potential targets.

**GO enrichment analysis and KEGG pathway enrichment analysis:** The results of GO analysis showed that in the BP category, differentially expressed genes were concentrated in the regulation of reactive oxygen species metabolic process, response to oxidative stress, cellular response to chemical stress, and...
others. Differentially expressed genes are enriched in vesicle lumen, cytoplasmic vesicle lumen, and secretory granule lumen in the CC category. Differentially expressed genes are enriched in tyrosine kinase activity, protein serine/threonine kinase activity, and phosphatase binding (Figure 5A). KEGG pathway analysis results showed that the differentially expressed genes involved EGFR tyrosine kinase inhibitor resistance and the PI3K-Akt signaling pathway (Figure 5B, Figure 6).

**DISCUSSION**

Effectiveness and safety of a formula used for CG treatment were evaluated by us. We also summarized the possible pharmacological mechanisms based on collecting as many medical records as possible. Before our study, at least two systematic reviews[42,43] focused on the efficacy of Chinese herbal medicine formulas as CG treatments. However, neither of these reviews included SLBZS as an experimental intervention, and there are no animal studies of SLBZS for CG.

Analysis of the 14 RCTs suggested that SLBZS reverses *H. pylori* seropositivity and recurrence rates in patients with CG more so than in western medicine. SLBZS formula treats CG based on the current evidence. There were insignificant heterogeneity and publication bias. The safety is not yet established. The study designs were not rigorous, and the GRADE assessment presented moderate and low quality. Therefore, large numbers of rigorously designed RCTs are required to obtain conclusive evidence for the effect and safety of SLBZS for CG.

CG is a common digestive system disorder characterized by an inflammatory condition of the gastric mucosa. CG also leads to mental and psychological disorders like interpersonal sensitivity and depression[44]. On the one hand, studies demonstrated that the link between gut flora and depression is strong[45-47], and gut peptides are essential regulators of microbiota-gut-brain signaling in health and stress-related psychiatric illnesses[45]. On the other hand, intestinal flora can be transformed by TCM compounds[48]. Chinese medicine can regulate the composition and metabolism of intestinal flora and regulate intestinal flora by affecting the secretion of brain-gut peptide and monoamine neurotransmitters, thus improving depression behavior[47-49]. Hence, the anti-inflammatory effect of regulating gut microbiota could represent a complementary and alternative direction for CG with depression symptoms.
Figure 5 GO analysis and Kyoto Encyclopedia of Genes and Genomes enrichment analysis. A: GO analysis of the critical targets of Shen-ling-bai-zhu san (SLBZS) in treatment for chronic gastritis (CG); B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the critical targets of SLBZS in treatment for CG.
Main pharmacological mechanisms

According to a study based on Chinese Medicine theory[50], the mechanism of TCM in treating CG is related to neuroprotective mechanisms, immune protective mechanisms, endocrine protective mechanisms, and other factors. A rat study showed that Xiangshaliujunzi decoction improved chronic atrophic gastritis symptoms by activating the TLR2, TLR4/MAPK/NF-κB/iNOS/NO signal pathway [51]. SLBZD reduced intestinal adenoma formation in adenomatous polyposis coli multiple intestinal neoplasia mice by suppressing hypoxia-inducible factor 1α-induced CD4 + CD25 + forkhead box P3 regulatory T cells[19]. Nevertheless, the mechanisms of SLBZS in CG have not been clarified.

In the present study, based on the network pharmacology analysis of drug and disease targets, a collateral relationship revealed the mechanism of SLBZS in the treatment of CG. First, we identified candidate target genes of SLBZS. Then, a protein interaction data network was generated, from which we obtained 36 related protein targets. The most protein targets included SRC, MAPK14, PPARG, and ERBB2. Critical GO entries were included regulation of reactive oxygen species metabolic process, response to oxidative stress, cellular response to chemical stress, protein tyrosine kinase activity, protein serine/threonine kinase activity, phosphatase binding, and others. Key signal pathways were identified in the KEGG enrichment analysis, primarily in EGFR tyrosine kinase inhibitor resistance, the PI3K-Akt signaling pathway, and others.

A study found that alterations in gastric cell stress-adaptive mechanisms due to H. pylori appear crucial during chronic infection[52]; therefore, response to oxidative stress of SLBZS to improve CG symptoms may determine the mechanism. In a future study, we will combine chemical analysis with network pharmacology to study the pharmacological effects of complex formulations comprehensively. The candidate target proteins and the formula’s active ingredients are predicted by analyzing the corresponding networks. The chemical ingredients may be fully identified through experiments to confirm their presence in the formula. Therefore, further animal and clinical experiments are needed for research and exploration.

Limitations

This study had many limitations: (1) Only small sample sizes Chinese-language RCTs were included, and there were some defects in research design that resulted in the low or moderate quality of evidence; (2) most studies had design flaws like it focused only on results without illustrating a specific implementation of the random method, blind method, and follow-up reporting; (3) despite using validated documents supporting effectiveness assessment criteria, our non-uniform efficacy evaluation approach might influence outcomes and results. It might be challenging to employ the same effectiveness assessment criteria for each trial, as these criteria varied with each update; (4) adverse effects and recurrence rates information is rare reported; (5) the dosage of SLBZS has not been standardized and unified, and therefore the reasonable dosage was difficult to determined; (6) the pharmacology mechanism is unclear, especially the specific analysis of active ingredients and side effects; and (7) conflicts of interest of study investigators or funders may influence the risk of bias due to missing results. None of our included studies clearly reported their Chinese herbal sources, particularly whether pharmaceutical companies provided support. It is difficult to determine whether there were conflicts of interest. Presentation of herb sources in future studies could help determine bias.

CONCLUSION

This meta-analysis included 14 RCTs and summarized the clinical efficacy and potential mechanisms of the Chinese herbal formula SLBZS in treating CG. However, the methodological quality of the studies was not high, the risk of relapses and adverse reactions was underreported, and related mechanisms lacked validation; therefore, rigorous RCTs and basic science studies should be designed further to determine a definitive association between SLBZS and CG.
Figure 6 Schematic diagram. A: Schematic diagram of main Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, EGFR tyrosine kinase inhibitor resistance; B: Schematic diagram of the main KEGG pathways, PI3K-AKT signaling pathway, arrows represent activation effect, T-arrows represent inhibition effect, and segments show activation or inhibition effects.
ARTICLE HIGHLIGHTS

Research background
The effects and safety of Shen-ling-bai-zhu san (SLBZS) are currently unclear.

Research motivation
A 2012 clinical practice guideline recommended SLBZ Powder for the Pattern of Spleen and Stomach Deficiency CG. The 2020 clinical guideline did not recommend SLBZS, possibly because of inadequate clinical evidence and pharmacological mechanisms. We designed our study to focus on evidence of efficacy and potential mechanisms. This controversy needed clarified.

Research objectives
To determine the clinical evidence and potential mechanisms of SLBZS for the treatment of CG.

Research methods
Evidence-based meta-analysis and network pharmacology methods.

Research results
Fourteen articles were eventually included, covering 1335 participants. SLBZS might treat CG by acting on related targets and pathways such as EGFR tyrosine kinase inhibitor resistance, the PI3K-Akt signaling pathway, and others.

Research conclusions
SLBZS might be useful in treating CG, but its long-term effects and specific clinical mechanisms keep unclear.

Research perspectives
More samples and high-quality clinical studies should be tested and verified in the next step.

FOOTNOTES

Author contributions: Jin W and Zhong J designed the protocol; this work was conducted by Jin W, Zhong J, Li QJ, Song Y, and Hou WW; the manuscript was drafted by Zhong J and revised by Li MF, Song SY, and Li CR; Jin W and Zhong J contributed equally to this work and should be regarded as co-first authors; all authors approved the final manuscript before submission.

Conflict-of-interest statement: There are no conflicts of interest to report.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Chen YL
L-Editor: Filipodia
P-Editor: Yu HG

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Global research trends in the field of liver cirrhosis from 2011 to 2020: A visualised and bibliometric study

Pei-Ling Gan, Shu Huang, Xiao Pan, Hui-Fang Xia, Xin-Yi Zeng, Wen-Sen Ren, Xian Zhou, Mu-Han Lv, Xiao-Wei Tang

BACKGROUND
Liver cirrhosis is the leading cause of liver-related mortality worldwide. It is currently a global health challenge.

AIM
This research intended to explore and analyse research trends and frontiers in this field during the last 10 years, providing new inspiration for clinical decision-making and scientific research.

METHODS
Publications on hepatic cirrhosis research were retrieved from the Web of Science Core Collection on April 4, 2021. Bibliometric visualisation was conducted through VOSviewer and CiteSpace.

RESULTS
The analytic research was based on original articles and reviews. A total of 7775 records of hepatic cirrhosis published from 2011 to 2020 were retrieved. In the past ten years, the number of related annual publications has increased significantly, especially in the United States and China. All publications were distributed among 109 countries. The United States contributed the most (21.95%) and was consistently the leading driving force, with a solid academic reputation in this area. The University of Barcelona distributed the most related articles (177
articles) and was cited the most frequently. The *Journal of Hepatology* ranked third in the top 10 journals, which has the highest impact factor (impact factor 2019 = 20.582). Jasmohan S. Bajaj was the most productive author (72 articles). Burst keywords (e.g., sofosbuvir, burden, care, sarcopenia, chronic liver failure, human gut microbiome, and nonalcoholic fatty liver disease) and a succession of reference citation bursts have provided clues about research frontiers in recent years.

**CONCLUSION**

This study identified developing trends in the evolution of liver cirrhosis to provide new inspiration for researchers.

**Key Words:** Liver cirrhosis; Bibliometric research; Research frontiers; VOSviewer; CiteSpace; Visualization

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**Core Tip:** This research explored and analyzed research trends and frontiers in this field during the last 10 years, further providing new inspiration for scientific research. We found sarcopenia, human gut microbiome, and nonalcoholic fatty liver disease are of particular interest in studies of cirrhosis. Treatment of diseases that cause cirrhosis, such as hepatitis C, is also a hot topic.

**INTRODUCTION**

Liver cirrhosis is a common clinical chronic progressive disease with high mortality caused by one or more factors. It is the fifth leading cause of adult deaths, the top cause of liver-related death worldwide [1], and the eighth of the primary diseases in economic cost[2]. Cirrhosis is a heterogeneous disease classified into two prognosis stages: compensated cirrhosis and decompensated cirrhosis[3]. In the early stage, due to the essential liver compensatory function, there are no visible symptoms. Later, the primary symptoms are liver function impairment and portal hypertension, and multiple systems are affected. Ascites, upper gastrointestinal haemorrhage, secondary infection, hepatic encephalopathy, canceration, and other complications are common in the late stage. Thus, cirrhosis is a high-burden treatment option for patients, health care systems, and the government.

Bibliometric research is a quantitative analytic method that employs mathematics and statistics to determine scientific activity[4]. It can help researchers identify the research focus and trends of a particular subject. In addition, the research results may be beneficial to future research. Professor Chen (Drexel University) created CiteSpace V, a Java-based information visualisation program for bibliometric analysis. Researchers may assess a discipline's evolution and identify frontier trends intuitively by providing numerous data in the form of knowledge maps[5]. CiteSpace has recently been used for bibliometric analysis in various fields; however, there is yet to be a bibliometric analysis of cirrhosis. In this study, we first used CiteSpace V to analyse articles on liver cirrhosis from 2011 to 2020 utilizing the Web of Science Core Collection (WoSCC) database, providing new inspiration for clinical decision-making and scientific research.

**MATERIALS AND METHODS**

**Data collection and search strategy**

Data were retrieved from the WoSCC on a single day, April 4, 2021. WoSCC offers extensive citation index information for over 8000 influential and famous journals globally. It is a comprehensive database, notably in natural science and medicine. The following were included in the search strategy: title = “cirrhosis,” database selected = Web of Science Core Collection, time span = 2011–2020. Only original articles and reviews were included. We obtained 7775 records for this study. A flowchart representing the retrieval strategies is shown in Supplementary Figure 1. In addition, high-quality references were retrieved and cited by the Reference Citation Analysis (https://www.referencecitation-analysis.com/) database.
Analysis tool
For bibliometric analysis, Microsoft Excel 2016, VOSviewer, and CiteSpace were chosen. Information on authors, journals, institutions, and countries may be integrated with these computer systems. Parameters such as article count, impact factor (IF), centrality, and occurrence/citation burst were utilized in this article. Productivity was measured by the number of articles published and was used to identify productive individuals or groups. The IF was obtained using Journal Citation Reports (JCR). The IF is a recognized metric for assessing a journal’s impact worldwide. The IF in this study was based on JCR (2019). The network visualisation maps were constructed using VOSviewer to examine the cooperative relationships between countries and institutions with highly cocited references. Coauthorship analysis identifies research output. We selected “countries” and “organizations” as the unit of analysis. CiteSpace V adopts a time slicing technique to create a timeline of network models and integrates these individual networks to produce an overview network for the systematic analysis of the relevant publications. In this study, we utilized CiteSpace V to conduct a cocitation analysis of the references and clusters. After that, a timeline view of cocited references was built. As a result, we were able to clarify the origin and period of certain clustering fields. These parameters help identify potential collaborative relationships in the field of liver cirrhosis. Furthermore, an occurrence burst denotes a word that appears often over a specific period, whereas a citation burst denotes a reference that is referenced frequently during a specific period. Keywords and references with the highest citation bursts were selected to demonstrate research hot spots and frontiers because they can identify whether relevant scholars have paid extensive attention to these areas in a specific period.

RESULTS
Publication output and temporal trend
A total of 7775 publications satisfied the search criteria. Table 1 lists the top-10 cited articles in descending order based on the number of citations. Figure 1A depicts the global distribution of yearly publications on cirrhosis research from 2011 to 2020. The overall worldwide publishing trend rose from 520 to 955 between 2011 and 2020. However, during the periods of 2015-2017 and 2019-2020, the number of publications showed a modest decrease. The annual publication trends of the top 10 academic output countries are presented in Figure 1B. The number of publications on cirrhosis published by the United States and China has increased remarkably within the last decade.

Distribution by country and institution
All of the publications come from 109 countries and 6902 institutions. The distribution of countries/regions that published no fewer than 100 papers is shown in Supplementary Figure 2. Table 2 contains detailed information on the top 10 countries. The United States had the most publications (1707 publications), followed by China (1672 publications) and Japan (711 publications). Among the top 10 countries, France exhibits high academic quality, and its citation/article ratio (46.67) was far greater than that of other listed countries.

Using VOSviewer, we constructed a network visualisation map for liver cirrhosis research publications to assess international collaborations. Collaborations between countries and institutions are depicted in Supplementary Figure 3. Nodes with higher co-occurrence were classified as the same colour. Nodes with similar colours formed one cluster, indicating that they had closer cooperative relationships. The width of the lines describes the magnitude of the collaboration. As shown, the United States had the highest total link strength, suggesting that it cooperated most with other countries worldwide. The country that collaborated the most with the United States was China. The most productive institutions are listed in Table 3. The University of Barcelona (177 publications) ranked first, followed by Virginia Commonwealth University (120 publications) and University College London (119 publications). The cluster coloured in green was led by the University of Barcelona, collaborating most with Virginia Commonwealth University. Furthermore, three of the top 10 institutions are in the United States, two are in China, and two are in Italy, suggesting that these three countries have many outstanding research groups in this field.

Distribution by journals and authors
Liver cirrhosis research articles were published in 1511 journals. The number of articles in the top 10 journals ranged from 119 to 259, accounting for 22.73% of the total (1767) (Supplementary Table 1). Among these journals, Liver International contributed the highest number of publications on cirrhosis research (259 publications, IF 2019 = 5.175), followed by Hepatology (242 publications, IF 2019 = 14.679) and Journal of Hepatology (212 publications, IF 2019 = 20.582). Journal of Hepatology had the highest IF, and its citation/article ratio (68.30) was far more than that of other listed journals. The most frequently cited journal was Hepatology (14922 citations). The following most frequently cited journals were Journal of Hepatology (14479 citations) and Liver International (5721 citations).
Table 1 The top 10 cited articles of Web of Science Core Collection bibliometrics in cirrhosis research field

<table>
<thead>
<tr>
<th>Rank</th>
<th>First author</th>
<th>Journal</th>
<th>Title</th>
<th>Number of citations (WoSCC)</th>
<th>Type of articles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moreau R</td>
<td>Gastroenterology 2013; 144(7): 1426-1437, 1437.e1-9</td>
<td>Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis</td>
<td>1113</td>
<td>Retrospective study</td>
</tr>
<tr>
<td>2</td>
<td>Marcellin P</td>
<td>Lancet 2013; 381(9665): 468-475</td>
<td>Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study</td>
<td>946</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>3</td>
<td>Kowdley KV</td>
<td>N Engl J Med 2014; 370(20): 1879-1888</td>
<td>Ledipasvir and Sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis</td>
<td>872</td>
<td>Prospective study</td>
</tr>
<tr>
<td>4</td>
<td>Qin N</td>
<td>Nature 2014; 513(7516): 59-64</td>
<td>Alterations of the human gut microbiome in liver cirrhosis</td>
<td>793</td>
<td>Retrospective study</td>
</tr>
<tr>
<td>5</td>
<td>Anstee QM</td>
<td>Nat Rev Gastroenterol Hepatol 2013; 10(6): 330-344</td>
<td>Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis</td>
<td>758</td>
<td>Review</td>
</tr>
<tr>
<td>9</td>
<td>Conti F</td>
<td>J Hepatol 2016; 65(4): 727-733</td>
<td>Early occurrence and recurrence of hepatocellular carcinoma in HCV-related cirrhosis treated with direct-acting antivirals</td>
<td>495</td>
<td>Prospective study</td>
</tr>
<tr>
<td>10</td>
<td>Chen Y</td>
<td>Hepatology 2011; 54(2): 562-572</td>
<td>Characterization of fecal microbial communities in patients with liver cirrhosis</td>
<td>450</td>
<td>Randomized controlled trial</td>
</tr>
</tbody>
</table>

WoSCC: Web of Science Core Collection.

Table 2 The top 10 countries that published articles on cirrhosis research

<table>
<thead>
<tr>
<th>Rank</th>
<th>Country</th>
<th>Number of articles</th>
<th>Number of citations</th>
<th>Citations per article</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>United States</td>
<td>1707</td>
<td>46238</td>
<td>27.09</td>
</tr>
<tr>
<td>2</td>
<td>China</td>
<td>1672</td>
<td>19452</td>
<td>11.63</td>
</tr>
<tr>
<td>3</td>
<td>Japan</td>
<td>711</td>
<td>11215</td>
<td>15.77</td>
</tr>
<tr>
<td>4</td>
<td>Italy</td>
<td>651</td>
<td>22183</td>
<td>34.08</td>
</tr>
<tr>
<td>5</td>
<td>Spain</td>
<td>486</td>
<td>19497</td>
<td>40.12</td>
</tr>
<tr>
<td>6</td>
<td>Germany</td>
<td>477</td>
<td>12862</td>
<td>26.96</td>
</tr>
<tr>
<td>7</td>
<td>England</td>
<td>458</td>
<td>19842</td>
<td>43.32</td>
</tr>
<tr>
<td>8</td>
<td>France</td>
<td>391</td>
<td>18247</td>
<td>46.67</td>
</tr>
<tr>
<td>9</td>
<td>South Korea</td>
<td>310</td>
<td>5077</td>
<td>16.38</td>
</tr>
<tr>
<td>10</td>
<td>India</td>
<td>302</td>
<td>3661</td>
<td>12.12</td>
</tr>
</tbody>
</table>

A total of 35653 authors contributed to the overall output. The most productive authors are listed in Supplementary Table 2. Jasmohan S. Bajaj published 72 articles, ranking first in the number of publications, followed by M. Eric Gershwin (70 articles) and Qi Xingshun (61 articles). Pere Gines had the highest citation/article ratio (89.27), followed by Paolo Angeli (74.07) and Guadalupe Garcia-Tsao (69.13).

Analysis of keywords
Figure 2 shows the top 25 keywords with the strongest occurrence burst. The occurrence burst, which demonstrated a sharp rise over a certain period, referred to frontier disciplines and dynamic changes in
Table 3 The top 10 institutions that published articles on cirrhosis research

<table>
<thead>
<tr>
<th>Rank</th>
<th>Institutions</th>
<th>Number of articles</th>
<th>Number of citations</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>University of Barcelona</td>
<td>177</td>
<td>11087</td>
<td>Spain</td>
</tr>
<tr>
<td>2</td>
<td>Virginia Commonwealth University</td>
<td>120</td>
<td>5826</td>
<td>United States</td>
</tr>
<tr>
<td>3</td>
<td>University College London</td>
<td>119</td>
<td>5134</td>
<td>England</td>
</tr>
<tr>
<td>4</td>
<td>Mayo Clinic</td>
<td>117</td>
<td>4074</td>
<td>USA</td>
</tr>
<tr>
<td>5</td>
<td>University of Padua</td>
<td>111</td>
<td>5430</td>
<td>Italy</td>
</tr>
<tr>
<td>6</td>
<td>Capital Medical University</td>
<td>109</td>
<td>646</td>
<td>China</td>
</tr>
<tr>
<td>7</td>
<td>University of California, Davis</td>
<td>102</td>
<td>4515</td>
<td>USA</td>
</tr>
<tr>
<td>8</td>
<td>Zhejiang University</td>
<td>102</td>
<td>2404</td>
<td>China</td>
</tr>
<tr>
<td>9</td>
<td>University of Copenhagen</td>
<td>99</td>
<td>1521</td>
<td>Denmark</td>
</tr>
<tr>
<td>10</td>
<td>University of Milan</td>
<td>98</td>
<td>5108</td>
<td>Italy</td>
</tr>
</tbody>
</table>

Figure 1 Trends in the number of publications and analysis of countries in the field of cirrhosis. A: The annual worldwide publication output; B: The annual publication output for the top 10 countries.

Analysis of references

In bibliometric research, reference analysis is an important indication. The pieces of literature with the strongest citation burst are considered to be the knowledge fundamentals of the research frontiers. Clusters along horizontal timelines are depicted in Figure 3, which is a timeline visualisation in CiteSpace. From left to right, each cluster is presented. The publication time legend is displayed at the top. The clusters were arranged vertically and in declining order of size. The largest cluster is presented at the top. Cocitation linkages are shown by the stained curves. Large-sized nodes were especially concerning since they were highly cited. The most cited references in a given year are displayed under each timeline. Clusters were numbered from 0. Cluster #0 was the largest cluster. As shown in the timeline overview, the largest cluster in this study was #0 acute kidney injury, indicating a significant research interest and direction in recent years. This was followed by #1 hepatitis C, #2 clonal selection theory, #3 hepatic encephalopathy, #4 Liver cirrhosis, and #5 variceal bleeding. Some clusters remained active until 2020. Figure 4 depicts cirrhosis-related references with the strongest citation burst during the last 10 years. Citation bursts until the end of 2020 were led by de Franchis et al (2015), who had the strongest burst (40.22), followed by Garcia-Tsao et al (2017), Tschantzis et al (2014), Vilstrup et al (2014),
DISCUSSION

Liver cirrhosis is a chronic disease with high mortality and is a serious public health problem, affecting more than 160 million people globally in 2017[8]. The most common cause is chronic viral hepatitis, specifically hepatitis B virus (HBV) and hepatitis C virus (HCV)[3,9]. Previous studies have shown that the prevalence of liver cirrhosis varies by region. East Asia has the most significant prevalence of liver cirrhosis. In contrast, Southern Latin America has the lowest incidence of 12.1%[9]. Furthermore, the worldwide incidence of liver cirrhosis is increasing continuously, and it ranks eighth in terms of economic cost among significant diseases[2], even though various public health measures have been implemented. As a result, to better comprehend the advanced research hotspots in the field of cirrhosis, a study is necessary.

From the retrieved data, we can easily conclude that the general research trend of liver cirrhosis has been growing, especially in China and the United States. Eight of the top 10 countries that most produce related research were developed countries, while 2 were developing countries. The United States had the most publications, followed by China and Japan, and India ranked last. In 2017, among individuals with prevalent liver cirrhosis worldwide, HBV infection accounted for approximately 28.72%. East Asia has the highest prevalence of liver cirrhosis. China had the world's greatest number of patients. Therefore, China and India rank among the top 10 countries in terms of the occurrence of liver cirrhosis and may be relevant for these potential causes. However, in terms of citations per article, although China temporarily ranked in the top position in number of articles, it still has a long way to go to improve the overall quality of articles. China must continue to increase research in this area and strengthen collaborations with other countries. In contrast, although only a few French articles were published, they were of high academic quality.
Nine of the top 10 institutions were universities, suggesting that universities are the most common research groups. Three of the top 10 institutions were from the United States (Virginia Commonwealth University, Mayo Clinic, and University of California, Davis); thus, the United States is the main domain in the field. Two of the top 10 institutions were from China (Capital Medical University and Zhejiang University), and two institutions were from Italy (the University of Padua and University of Milan). According to the findings of this analysis, the three countries have numerous great research groups in this area.

In this study, we found some great research works from some excellent authors. They made significant contributions to this discipline. For example, Guadalupe Garcia-Tsao ranked 7th among the top 10 authors, and her practice guideline of portal hypertensive bleeding in cirrhosis also ranked among the top 10[10]. Her study effort provides academic researchers and clinical professionals with a better and deeper understanding of risk stratification, diagnosis, and management of portal hypertensive bleeding in liver cirrhosis. Moreau et al.[11] analysed data from patients with cirrhosis and acute decompensation (AD) to establish diagnostic criteria for acute-on-chronic liver failure (ACLF), which he discovered was distinct from AD. In addition, his research demonstrated that ACLF mortality is linked to loss of organ function and high leukocyte counts and that ACLF is particularly severe in individuals with no prior history of AD.

CiteSpace V was used to detect keyword bursts in this study. These statistics are potentially valuable in forecasting research frontiers. The keywords “sofosbuvir,” “burden,” “care,” “sarcopenia,” “chronic liver failure,” “human gut microbiome,” and the “NAFLD” are expected to appear often in the following years, indicating emerging trends. The top five cirrhosis frontiers are as follows: (1) Sarcopenia: The skeletal muscle is the body’s largest organ, and atrophy comes from a change in the balance of protein production and degradation towards protein breakdown. Primary sarcopenia is a loss of skeletal muscle strength, mass, or physical function owing to ageing, whereas secondary sarcopenia is recognized as a loss of skeletal muscle strength, mass, or physical function due to underlying disorders. Liver cirrhosis is one of the representational disorders that might be complicated with secondary sarcopenia. In patients with cirrhosis, muscle mass loss worsens as their liver reserve deteriorates. Sarcopenia and frailty have a negative impact on clinical outcomes and prognosis. It has been convincingly demonstrated that patients with cirrhosis, sarcopenia, and frailty have a lower quality of life and survival, more cirrhotic complications and infections, and poorer outcomes following liver transplant surgery[12-14]. As a result, emphasis must be placed on early detection, active diagnosis, and treatment. Consequently, further investigations in the context of assessment criteria for sarcopenia and frailty are warranted to deepen our current knowledge of sarcopenia and frailty in patients with cirrhosis; (2) Chronic liver failure: ACLF, a clinical syndrome in patients with chronic liver disease, is more often discussed clinically. It is associated with multiple organ failure and increased short-term mortality. It is a significant cause of mortality in people with cirrhosis. Although the precise causative mechanism is

Figure 3 A timeline visualization of the largest clusters.
unknown, systemic inflammation plays a critical role in its pathogenesis, and the strength of this inflammatory response paralleled the severity of ACLF. Aside from organ failure and a very high risk of short-term death, patients with ACLF exhibited other characteristics that separated them from non-ACLF patients. They were, for example, younger, had more alcoholic cirrhosis and less HCV-related cirrhosis, and mainly had a higher frequency of active alcoholism and severe bacterial infections [11]. More research for new findings into the mechanisms underlying ACLF, risk prediction models, therapeutic targets, and liver transplantation for ACLF is needed; (3) Sofosbuvir: An oral nucleotide analogue inhibitor of the HCV-specific NS5B polymerase has been approved to treat chronic HCV infection. A study showed that ledipasvir-sofosbuvir for eight weeks was associated with a high rate of sustained virologic response among previously untreated patients with HCV genotype 1 infection without cirrhosis [15]. A recent article published in *The Lancet Gastroenterology & Hepatology* stated that virdiasvir plus sofosbuvir was effective in patients with chronic HCV infection. Furthermore, by offering a new affordable, simple, and efficacious public health tool for large-scale implementation, this treatment has the potential to eradicate HCV-related morbidity and mortality [16]; (4) Human gut microbiome: The function of gut microbiota in human health and disease has recently received much attention. Several chronic illnesses have been linked to the gut microbiota, such as obesity [17-19], inflammatory bowel disease [20], diabetes mellitus [21], and NAFLD [22]. Through the hepatic portal and bile secretion systems, the liver interacts directly with the gut. Enteric dysbiosis, namely, the translocation of bacteria and their products over the gut epithelial barrier, is involved in the development of liver cirrhosis. However, the phylogenetic and functional composition alterations in the human gut microbiota associated with this progression remain obscure [23]. Furthermore, research suggests that microbiome manipulation to eliminate manganese and reduce GABA levels in the gut might provide a novel therapeutic approach for treating hepatic encephalopathy [24]. In addition, novel probiotics may be helpful in the prevention of the aggravation of liver cirrhosis. More broadly, microbiome manipulation may offer up new pathways for the treatment of liver cirrhosis. A combination of microbial genes distinguishes patients with liver cirrhosis from healthy individuals with high specificity. This might pave the way for a novel method of monitoring and preventing liver cirrhosis; and (5) NAFLD: NAFLD is a
progressive liver disease spectrum encompassing simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and, eventually, cirrhosis. NAFLD is closely associated with characteristics of metabolic syndrome such as abdominal obesity, insulin resistance, glucose intolerance or type 2 diabetes (T2DM), and atherogenic dyslipidaemia\([25]\). Over the last few decades, people’s lifestyles have become more sedentary, and dietary habits have changed, leading to an increase in the prevalence of obesity and insulin resistance\([26,27]\). In light of this, NAFLD quickly became the most common cause of abnormal liver biochemistry in both developed and developing countries. Several prior epidemiological studies have identified a strong association between NAFLD and an elevated risk of developing T2DM. In addition, patients with NAFLD, whether adults or adolescents, have several risk factors for cardiovascular disease. A notable minority proportion of NAFLD patients proceed to a more severe disease characterized by NASH and fibrosis and cirrhosis or, in some circumstances, progress to hepatocellular carcinoma\([28]\). The mechanisms linking NAFLD to the diseases mentioned above and the current pharmacological treatments for NAFLD need to be further explored.

The articles with the strongest citation burst are potentially valuable for exploring research frontiers. In this study, #0 acute kidney injury was the largest cluster, and #3 hepatic encephalopathy remained active until the most recent publication year for a cited reference. References with the top three citation bursts were as follows: (1) de Franchis et al\([29]\) contributed to the writing of expanding consensus in portal hypertension, further elaborating the stratifying risk and individualizing care for portal hypertension; (2) Garcia-Tsao et al\([10]\) coauthored a practice guideline and elaborated the risk stratification, diagnosis, and management of portal hypertensive bleeding in cirrhosis; and (3) Tsochatzis et al\([30]\) reviewed the current understanding of cirrhosis as a dynamic process and outlined therapeutic options for preventing and treating complications of cirrhosis based on the subclassification in clinical stages. Meanwhile, we proposed a new concept for managing patients with cirrhosis and the challenge in the 21st century.

**Limitations**

To the best of our knowledge, this study was the first bibliometric analysis of cirrhosis in the past ten years. This study, however, has certain limitations. First, we considered the WoSCC database a reputable and reliable service for publications and citations; hence, we extracted data only from it. This may restrict the coverage of all available articles and result in a reduced number of documents included in the analysis. Second, the search method may also have been insufficient because we only searched for publications with the phrase 'cirrhosis,' which may have resulted in a paucity of papers due to other terminology. Finally, we analysed the data selectively. We mainly utilized a quantitative analysis approach, while little emphasis has been paid to the qualitative aspects of this study. As a result, certain critical points and details may have been missed. All of the considerations mentioned above may lead to bias in the results. Hence, the results should be interpreted with caution.

**CONCLUSION**

This study might aid researchers in identifying new trends in cirrhosis from 2011 to 2020, thus, providing new inspiration for scientific research.

**ARTICLE HIGHLIGHTS**

**Research background**

Liver cirrhosis is a common clinical chronic progressive disease with high mortality caused by one or more factors.

**Research motivation**

Identifying new trends in cirrhosis from 2011 to 2020, thus, providing new inspiration for scientific research.

**Research objectives**

This research intended to explore and analyse research trends and frontiers in this field of cirrhosis during the last 10 years.

**Research methods**

Using VOSviewer and CiteSpace, assess a discipline’s evolution and identify frontier trends intuitively by knowledge maps.
Research results
The general research trend of liver cirrhosis has been growing, especially in China and the United States. The keywords “sofosbuvir,” “burden,” “care,” “sarcopenia,” “chronic liver failure,” “human gut microbiome,” and the “NAFLD” are expected to appear often in the following years, indicating emerging trends. The top five cirrhosis frontiers are Sarcopenia, Chronic liver failure, Sofosbuvir, Human gut microbiome and Nonalcoholic fatty liver disease.

Research conclusions
This study identified developing trends in the evolution of liver cirrhosis to provide new inspiration for researchers.

Research perspectives
The top five cirrhosis frontiers are sarcopenia, chronic liver failure, sofosbuvir, human gut microbiome and nonalcoholic fatty liver disease.

FOOTNOTES
Author contributions: Gan PL, Huang S and Pan X contributed equally to this work; Gan PL, Zhou X, Lv MH and Tang XW designed the research study; Gan PL, Huang S, Pan X, Xia HF and Zeng XY performed the research; Pan X and Ren WS contributed analytic tools; Gan PL, Huang S and Pan X analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

Conflict-of-interest statement: We declared that no conflicts of interest or financial ties to disclose.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Yuan YY

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Ectopic bronchogenic cyst of liver misdiagnosed as gallbladder diverticulum: A case report

Cheng-Ji Dong, Ru-Ming Yang, Qi-Long Wang, Qing-Yuan Wu, Da-Ji Yang, De-Cai Kong, Ping Zhang

Abstract

BACKGROUND
Ectopic bronchogenic cysts are a type of congenital cystic tumor that are extremely difficult to diagnose and can be ectopically located in various organs, with the possibility of malignant transformation. Here we report a case of an ectopic bronchogenic cyst in the liver initially misdiagnosed as a gallbladder diverticulum.

CASE SUMMARY
The patient was a middle-aged woman whose chief complaint was intermittent pain in the upper abdomen. Imaging examination revealed a cystic space in the left inner lobe of the liver. She was admitted to our hospital for treatment. Based on abdominal examination and imaging findings, the initial diagnosis was gallbladder diverticulum with cholestasis combined with chronic cholecystitis. However, following intraoperative observations and postoperative pathologic assessment, the diagnosis was revised to ectopic bronchogenic cyst of the liver.

CONCLUSION
Radiologists, hepatobiliary and pancreatic surgeons, gastrointestinal surgeons, urologists, and even neurosurgeons should be aware and consider a possible diagnosis of ectopic bronchogenic cysts, especially when other types of cyst, cystadenoma, and other diseases are excluded. The disease and its complications should be detected and correctly diagnosed and treated as early as possible in order to avoid adverse outcomes.
INTRODUCTION

A bronchogenic cyst is a rare congenital cystic tumor that can generally be classified as one of three types—namely, intrapulmonary, mediastinal, and heterotopic (the most rare) [1-3]. Bronchogenic cysts often develop ectopically in the neck, retroperitoneum, and stomach wall; very few cases of ectopic cysts in the liver have been reported [4]. Ectopic bronchogenic cysts often have no specific clinical manifestations, imaging characteristics, or changes in laboratory indices, making preoperative diagnosis extremely difficult. The cysts are typically discovered during routine physical examinations or because of complications resulting from the compression of surrounding organs or tissues. Here we report a case of an ectopic bronchogenic cyst in the liver that was preoperatively diagnosed as cholecystic diverticulum with cholestasis.

CASE PRESENTATION

Chief complaints
A 40-year-old woman presented with intermittent upper abdominal pain.

History of present illness
Physical examination at the primary hospital revealed a space-occupying lesion above the gallbladder in the left inner lobe of the liver that was complicated by chronic cholecystitis. The patient was transferred to our hospital for further treatment.

History of past illness
The patient was physically fit.

Personal and family history
Her family history was noncontributory.

Physical examination
A physical examination of the abdomen showed no muscle tension, tenderness, or rebound pain. No abdominal mass was detected; the liver was not touched under the ribs, and the Murphy sign was positive.

Laboratory examinations
Laboratory examinations including routine blood and blood coagulation, liver function, biochemistry, tumor markers, and infection markers revealed no abnormalities.

Imaging examinations
Abdominal color Doppler ultrasound showed a cystic echo beside the gallbladder in the left inner lobe of the liver with a size of 3.2 cm × 1.6 cm. The boundary was clear, the ultrasound transmission was poor, a bile mud echo was observed, there was no blood flow signal, and there appeared to be a...
connection with the neck of the gallbladder. Gallbladder diverticulum with cholestasis and chronic cholecystitis was suspected. Hepatobiliary, pancreatic, and splenic multi-slice plain computed tomography (CT) scanning and 3-phase enhanced scans showed an elliptical low-density shadow above the gallbladder in the left inner lobe of the liver, with a CT value of about 55 HU. Intrahepatic bile ducts were not dilated. The gallbladder was not enlarged but the wall was slightly thickened, and there was no abnormal density in the cavity. The cystic space-occupying lesion above the gallbladder in the left inner lobe of the liver strongly suggested gallbladder diverticulum with cholestasis. Magnetic resonance cholangiopancreatography (MRCP) was performed to determine the relationship between the lesion and the gallbladder. There was an oval abnormal signal in the gallbladder fossa area about 2.9 cm × 2.0 cm in size. T1-weighted imaging (T1WI) and T2-weighted imaging (T2WI) and diffusion-weighted imaging showed a high-signal shadow and the apparent diffusion coefficient was consistent with a slightly high-signal shadow; the edges of the shadow were thin, smooth, and clear, and there was no enhancement at the lesion center. The lesion appeared to be connected to the cystic duct given the limited fluid signal shadow in the gallbladder fossa area of the intrahepatic lobe (Figure 1).

**FINAL DIAGNOSIS**

Based on the patient’s symptoms, abdominal examination, and imaging findings, the initial diagnosis was gallbladder diverticulum with cholestasis combined with chronic cholecystitis. However, based on intraoperative findings and postoperative pathologic results, the diagnosis was later confirmed as ectopic bronchogenic cyst in the liver.

**TREATMENT**

The patient underwent laparoscopic cholecystectomy and cystectomy of the left inner lobe of the liver. During the operation, it was observed that the cyst and gallbladder were connected to a pedicle but not to each other. Dark green bile was drained from the gallbladder, and light yellow turbid fluid was observed inside the cyst (Figure 2).

**OUTCOME AND FOLLOW-UP**

After the operation, we carried out a pathologic examination on the excised specimen. The outer part of the cyst was connected to the gallbladder; the volume was 2 cm × 2 cm × 2 cm, the wall thickness was 0.1-0.2 cm, and the inner wall was smooth and light brown. The cyst wall was fibrous under microscopic examination, with visible muscle tissue. Part of the mucosal epithelium on the inner surface was absent, and the remaining inner surface was partly lined with pseudostratified ciliated columnar epithelium and mucous epithelium. Serous and mucous glands were observed in the lamina propria, consistent with bronchogenic cysts. The intraoperative findings combined with histopathologic examination refuted the preoperative diagnosis of gallbladder diverticulum complicated by cholestasis (Figure 3). No recurrence was found in the color Doppler ultrasonography 6 mo after the operation.

**DISCUSSION**

Ectopic bronchogenic cysts can occur at any age, with similar prevalence in men and women. There are few specific manifestations but clinical symptoms can arise when a cyst compresses nearby organs or tissues. For example, a patient developed new abdominal pain and gross hematuria that may have been caused by the aggravation of a lump originating from the left adrenal gland[5]; the pain was intermittent, which may have been related to chronic cholecystitis. Similarly, ectopic bronchogenic cysts have no special imaging findings. Magnetic resonance imaging (MRI) examinations often show isointense shadows on T1WI and high-intensity shadows on T2WI that are not enhanced by the contrast agent. However, in some patients, both T1WI and T2WI show significant enhancement[6]. In our patient, T1WI showed a slightly high-signal shadow and T2WI showed a high-signal shadow; a retrospective analysis of the MRI findings was more suggestive of ectopic bronchogenic cysts, which are associated with elevated levels of the tumor markers carcinoembryonic antigen, carbohydrate antigen (CA)19-9, and CA72-4[7,8].

In countries where bronchogenic cysts are endemic, they are often associated with hydatid disease[9]. The diagnosis and differential diagnosis of ectopic bronchogenic cysts is extremely difficult and they are often confused with other types of cyst and serous or mucinous cystadenoma. The pathogenesis of ectopic bronchogenic cysts in the abdominal cavity is complex and diverse. It has been proposed to
Figure 1 Ectopic bronchogenic cyst in a 40-year-old woman. The location of the cyst is indicated by an orange arrow. Magnetic resonance cholangiopancreatography (MRCP) showed that the cyst was locally connected to the cystic duct. A: Hepatobiliary and pancreatic enhanced computed tomography (CT) arterial phase; B: Hepatobiliary and pancreatic enhanced CT venous phase; C and D: MRCP; E: T1-weighted imaging showed a slightly high signal shadow; F: T2-weighted imaging showed a high signal shadow.

Figure 2 Gross examination of the surgically excised ectopic bronchogenic cyst. A: The gallbladder and cyst were removed under laparoscopy, revealing the smooth wound surface (orange arrow) connecting the cyst and liver; B: The inner wall of the cyst. The cyst contained light yellow turbid liquid; C: The cyst was connected to the gallbladder via a pedicle; D: Inner wall of the cyst.

originate in the embryonic foregut, which develops from the primordial malformed trachea and bronchial tree in the early embryo; bacteria shed from this structure migrate to the caudal side of the foregut, which forms the abdominal organ or retroperitoneal bronchus cyst. The thoracic and abdominal cavities are thought to be connected in the early embryonic stage. At 6 wk of gestation, the pleura and
Figure 3. Histopathologic examination by hematoxylin and eosin staining. The cyst wall was fibrous and muscle tissue was visible. The mucosal epithelium of the inner surface of the cyst wall was partially detached, and the remaining inner surface was partly lined with pseudostratified ciliated columnar epithelium (orange arrow) and mucous epithelium. Serous and mucous glands were observed in the lamina propria (green arrow). A: 4×; B: 10×; C: 20×; D: 40×.

CONCLUSION

Ectopic bronchogenic cysts are a congenital cystic tumor that are extremely difficult to diagnose and can be ectopically located in various organs, and have a risk of malignant transformation. Radiologists, hepatobiliary and pancreatic surgeons, gastrointestinal surgeons, urologists, and even neurosurgeons should be aware of these cysts. During diagnosis and treatment of a suspected cyst or cystadenoma, the possibility of ectopic bronchogenic cysts should be considered. The disease and its complications should be detected and diagnosed and treated as early as possible to avoid adverse consequences.

FOOTNOTES

Author contributions: Zhang P and Dong CJ performed the surgery, reviewed the literature, and contributed to manuscript drafting; Dong CJ, Yang RM, and Wang QL wrote the manuscript; Dong CJ, Wu QY, Yang DJ, and Kong...
DC were involved in the clinical management of the patient; all authors gave final approval for the submitted version of the manuscript.

Informed consent statement: Written, informed consent was obtained from the patient for publication of this report.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

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Prediction of moderately severe and severe acute pancreatitis in pregnancy: Several issues

Qun-Ying Yang, Jian-Wen Hu

LETTER TO THE EDITOR

We were pleased to read the high-level article published by Yang et al.[1]. In this article, the authors developed and validated a nomogram with good accordance for the prediction of moderately severe and severe acute pancreatitis in pregnancy (MSIP). The authors reported a nomogram that incorporated numerous blood indices for albumin, lactate dehydrogenase, triglyceride, and cholesterol levels, thus facilitating the early individualized prediction of the severity of acute pancreatitis in pregnancy.

Core Tip: This is a comment on a study involving the development and validation of a prediction model for moderately severe and severe acute pancreatitis in pregnancy. We believe that the role of cholesterol as a predictor should be more clearly described.

Citation: Yang QY, Hu JW. Prediction of moderately severe and severe acute pancreatitis in pregnancy: Several issues. World J Gastroenterol 2022; 28(33): 4926-4928

URL: https://www.wjgnet.com/1007-9327/full/v28/i33/4926.htm
(APIP). This study is of great significance for the clinical management of APIP. However, in our opinion, this article has some problems that need to be discussed further.

First, we found that some data that were not suitable for this article. In the result sections, we noticed that 134 patients were classified as having mild acute pancreatitis in pregnancy (MAIP) and 56 as having MSIP. However, Figure 1 (https://www.wjgnet.com/1007-9327/full/v28/i15/WJG-28-1588-g001.htm) and Table 1 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T1.htm) showed that the number of patients with MAIP and MSIP was 136 and 54, respectively. This inconsistency should be addressed.

In addition, there are problems associated with the statistical analyses in that the methods used for statistical analyses should be described in more detail. In Table 1 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T1.htm) and Table 2 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T2.htm), the authors should provide more accurate statistical values, including Student’s t-values or χ² values, instead of just providing P values. Most of the variables mentioned by the authors in Table 1 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T1.htm) and Table 2 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T2.htm), such as cholesterol and platelets, are not labeled with units of measurement. Moreover, P-values were not listed in the statistical results for “trimester of pregnancy on admission”.

In the Discussion section, the authors mentioned that hypercholesterolemia is a known risk factor for cardiovascular diseases. In fact, high-density lipoprotein (HDL) and non-HDL cholesterol have opposite associations with cardiovascular diseases[2], and plasma HDL cholesterol concentrations correlate negatively with the risk of cardiovascular diseases[3]. The authors mentioned that cholesterol is a predictive factor for MSIP, and the cholesterol levels of patients with MAIP and MSIP were 7.34 ± 5.63 and 12.80 ± 6.64, respectively, in Table 1 (https://www.wjgnet.com/1007-9327/full/v28/i15/1588-T1.htm). The authors mentioned some previous studies in their Discussion section. We took a close look at these studies and found that only one recent study[4] showed that low levels of total cholesterol (TC) and high TC within 24 h of admission were independently associated with an increased risk of severe acute pancreatitis. Other studies[5-7] have suggested that serum levels of HDL cholesterol are inversely correlated with disease severity in patients with predicted severe acute pancreatitis. However, this study showed that cholesterol is a predictive factor for MSIP but not HDL. This is a confusing statement. We believe that the present study is inconsistent with previous studies and that the role of cholesterol as a predictor should be more clearly described.

FOOTNOTES

Author contributions: Yang QY reviewed the literature and contributed to manuscript drafting; Hu JW was responsible for the revision of the manuscript for important intellectual content; all authors issued final approval for the version to be submitted.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Fan JR

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