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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 28; 27(44): 7582-7738

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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INDEXING/ABSTRACTING

The *WJG* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for *WJG* as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Yu-Jie Ma*; **Editorial Office Director:** *Ze-Mao Gong*.

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, Subrata Ghosh

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

PUBLICATION DATE
November 28, 2021

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

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https://www.wjgnet.com/bpg/gerinfo/242

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ONLINE SUBMISSION
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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 28; 27(44): 7582-7596

DOI: 10.3748/wjg.v27.i44.7582

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

FRONTIER

Orexins: A promising target to digestive cancers, inflammation, obesity and metabolism dysfunctions

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Author contributions: Couvineau A, Voisin T, Blais A have equally contributed in writing of the manuscript; Couvineau A, Voisin T, Nicole P, Gratio V and Blais A have read and approved the final version of manuscript.

Conflict-of-interest statement: Authors don't have any conflict of interest.

Supported by INSERM U1149/The Inflammation Research Center, Inserm Transfert, The Institut National du Cancer, No. 2013-213; Ligue Nationale Contre le Cancer, No. R16020HH, GB/MA/CD/EP-12062; and AgroParisTech (INRAE and Université Paris-Saclay).

Country/Territory of origin: France

Specialty type: Biology

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

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): 0

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Abstract

Hypothalamic neuropeptides named hypocretin/orexins which were identified in 1998 regulate critical functions such as wakefulness in the central nervous system. These past 20 years had revealed that orexins/receptors system was also present in the peripheral nervous system where they participated to the regulation of multiple functions including blood pressure regulation, intestinal motility, hormone secretion, lipolyze and reproduction functions. Associated to these peripheral functions, it was found that orexins and their receptors were involved in various diseases such as acute/chronic inflammation, metabolic syndrome and cancers. The present review suggests that orexins or the orexin neural circuitry represent potential therapeutic targets for the treatment of multiple pathologies related to inflammation including intestinal bowel disease, multiple sclerosis and septic shock, obesity and digestive cancers.

Key Words: Orexin; Neuropeptide; G-protein coupled receptor superfamily; Inflammation; Metabolic syndrome; Cancer

Core Tip: Twenty years ago, hypothalamic orexin peptides hypocretin-1/orexin-A and hypocretin-2/orexin-B) and their receptors were identified. They belong to the G-protein coupled receptor superfamily. Orexins and their receptors were involved in the regulation of many functions in the central nervous system: the regulation of wakefulness, drug addictions, food consumption, energy homeostasis and stress.

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

Received: April 20, 2021
Peer-review started: April 20, 2021
First decision: June 13, 2021
Revised: June 22, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 28, 2021

P-Reviewer: Li K
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

However, various biological effects have been also identified in the peripheral nervous system including endocrine and cardiovascular functions. Orexins/orexin receptors have been shown to play a major role in various peripheral diseases encompassing chronic inflammation and cancers. The present review focuses on the impact of orexin exogenous administration, in various pathophysiological conditions including digestive cancers, intestinal bowel disease, septic shock, multiple sclerosis and metabolic syndrome.

Citation: Couvineau A, Voisin T, Nicole P, Gratio V, Blais A. Orexins: A promising target to digestive cancers, inflammation, obesity and metabolism dysfunctions. *World J Gastroenterol* 2021; 27(44): 7582-7596
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7582.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7582

INTRODUCTION

Toward the end of the 1990s, two independent groups managed respectively by J.G. Sutcliffes and M. Yanagisawa have discovered two new hypothalamic neuropeptides which are produced by the same precursor encoded by a single gene[1,2]. One of these two seminal publications co-led by Luis de Lecea and Thomas Kilduff's was based on subtractive cDNA cloning strategy allowing the identification of the hypocretin-1 and hypocretin-2, a contraction between "hypothalamus" corresponding to the location of orexins production and "secretin", one hormone having a slight amino acid homology with hypocretins[1]. At the same time, the Takeshi Sakurai's publication based on the identification of orphan G protein-coupled receptors (GPCRs) by screening with purified unknown peptides from brain extracts has allowed the identification of both hypothalamic orexin-A (OxA) and orexin-B (OxB) referring to the Greek term meaning "appetite" because these two neuropeptides induced feeding after intraventricular brain injection[2]. These two neuropeptides identified by Sutcliffes's group and Yanagisawa's group turned out to be identical. It should be noted that the actual current use assigns the term "hypocretin" for the gene species and "orexins" for the protein species. From this discovery, the two orphans GPCRs have been identified as hypocretin (Hcrt)/orexin receptor 1 (OX1R) and Hcrt/orexin receptor 2 (OX2R) which belong to the large class A rhodopsin-like subfamily of GPCRs[3]. To elucidate the role of orexins in feeding, the Yanagisawa's group has developed a knock out mice invalidated for the hypocretin gene. This model has revealed that the regulation of feeding and appetite were not the major physiologic role of orexins[4]. However, the absence of orexins secretion in this model, led to cataplectic attack symptoms[4]. Moreover, the invalidation of OX2R induces sleep attack whereas the OX1R invalidation led to the sleep disturbance characterized by narcolepsy[5].

The role of the orexins/OXR system was firstly widely studied in the central nervous system (CNS) (Figure 1). Many publications have demonstrated and confirmed that the orexin main role in the CNS was to maintain wakefulness[6]. The absence of orexins production, associated to the loss of orexin neurons, in human CNS induce narcolepsy with cataplexy (Narcolepsy type I). The impact of orexin on sleep regulation had led to the development, by the pharmaceutical industries and the academic laboratories, of orexin receptor-targeting molecules, mainly antagonists, able to regulate the wake-sleep cycle for insomnia treatment[7]. A growing number of antagonists have been developed and classified into two categories: the single orexin-receptor antagonists (SORAs) including selective OX1R antagonist (SORA1s) and OX2R antagonist (SORA2s) and dual orexin-receptor antagonists (DORAs). Recently, two of these antagonists named suvorexant and lately lemborexant were approved by the U.S. Food and Drug Administration in insomnia treatment[8,9]. Moreover, the central action of orexins regulates food intake, energy homeostasis, reward seeking, stress, motivation and drug addictions[10,11] (Figure 1) which included the addiction to cocaine, opioids, amphetamines, cannabis, alcohol and nicotine[12]. Despite the major role of orexins in CNS, these two neuropeptides were also studied, to a lesser extent, in the peripheral nervous system (PNS). In various peripheral organs including the adrenal glands, kidney, cardiovascular system, reproductive tract, adipose tissue and digestive tract, orexins also acted as regulators (Figure 1)[13]. The relatively low

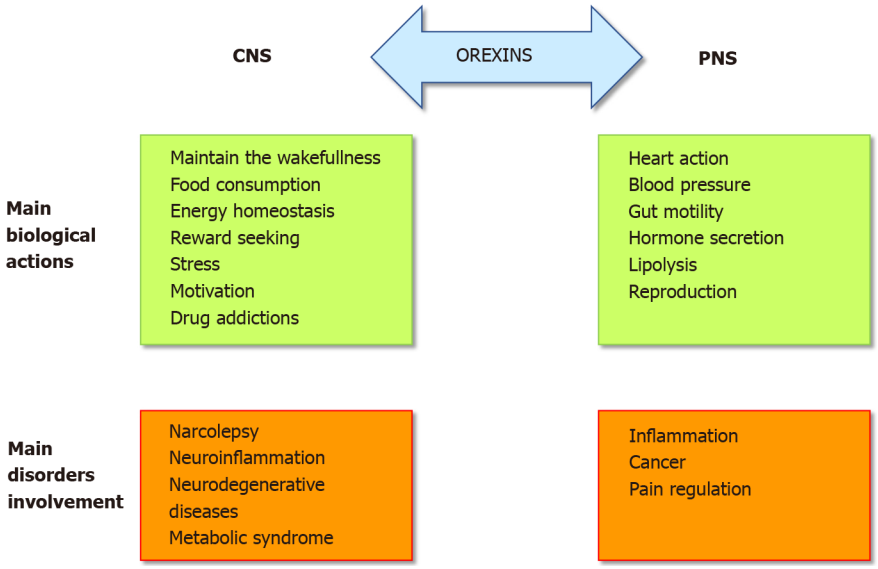

Figure 1 Pathophysiological roles of orexins/orexins receptors system. CNS: Central nervous system; PNS: Peripheral nervous system.

extensive studies of the roles of orexins in the PNS support that their actions were not fully elucidated and controversial[10]. In the digestive tract, orexin has been detected in neurons of the enteric nervous system (ENS) and in the enteroendocrine gut cells [14]. The presence of orexin in enteroendocrine cells supported the idea that this neuropeptide is involved in nutrition and energy homeostasis. Moreover, the use of different antibodies directed against OxA, OxB and prepro-orexin revealed an orexin-immunoreactivity in ENS[15]. Orexins modulate motility and orexin level was regulated by fasting[16]. In the pancreas, OxA was present in pancreatic islets, immunoreactivity being detected in pancreatic beta cells and potentially in alpha cells [14]. In humans, OX1R but not OX2R was also detected in pancreatic islets[17]. Although some studies support the role of OxA in the control of insulin secretion, this role remains conjectural[14]. However, the majority of these studies seemed to show that OxA directly or indirectly *via* the inhibition of glucagon release, regulated the insulin secretion[14]. It should be noted that OxB was also detectable in beta cells[17] but nothing is known about its role in the pancreas. If the presence of orexins in CNS, PNS and ENS was well established, the circulating level of these neuropeptides in healthy human blood was very low (about 2 to 50 pM) which is not enough to activate orexin receptors[18,19].

These biological effects were mediated through two orexin receptor subtypes, OX1R and OX2R which were coupled to Gq protein[10]. The interaction of orexins with its receptors led to the intracellular calcium release involving the phospholipase C (PLC) (Figure 2). Some reports have revealed that orexins were also able to activate the cAMP, PI3K/Akt, JNK and MAPK/Erk1/2 signaling pathways (Figure 2)[13]. The crystallographic structure of OX1R and OX2R associated to the suvorexant antagonist was reported[20,21]. Recently, the structure of OX2R complexed with OxB has been reported, suggesting that the molecular mechanisms which govern the activation or inactivation of receptors were located in the OX2R orthosteric site[22].

In pathological conditions, an abnormal expression of OX1R was observed in human peripheral organs. The presence of an ectopic expression of OX1R in intestinal bowel disease (IBD) including Crohn's disease and ulcerative colitis, in pancreatitis and digestive cancers as colon, pancreas and liver cancers, has been demonstrated[3]. The role of the orexin system in various human pathologies such as narcolepsy[23], neurodegenerative diseases (Alzheimer's disease)[24], ischemia[25], oxidative stress [26], chronic inflammation including IBD[10], multiple sclerosis[10] and metabolic syndrome[27] but also cancers[3], highlighted its potential therapeutic importance (Figure 1). In this context, the present review summarizes the impact of orexins and their receptors in chronic inflammation (*i.e.*, ulcerative colitis, multiple sclerosis, septic shock and metabolic syndrome) and cancers.

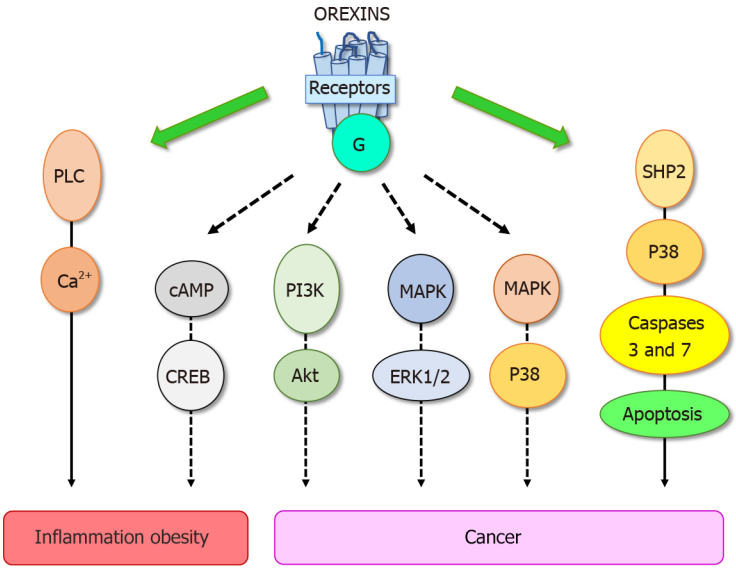

Figure 2 Main signaling pathways activated by orexins/orexins receptors system involved in peripheral diseases. PLC: Phospholipase C; cAMP: Cyclic adenosine monophosphate; CREB: C-AMP response element-binding protein; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; MAPK: Mitogen-activated protein kinase; ERK1/2: Extracellular signal-regulated kinase 1 and 2; P38: Mitogen-activated protein kinase; SHP2: Src homology 2 domains of Src homology 2-containing phosphatase 2.

OREXINS AND DIGESTIVE CANCERS

Despite the constant progress of the therapeutic arsenal, cancer is still the second causes of death worldwide[28]. To date, the treatment range options include surgery, chemotherapy, radiotherapy, hormonotherapy, antibody therapy, gene therapy, immunotherapy which integrate recent treatments based on anti-PDL-1 and CAR-T cells[29,30]. Digestive cancers, including colorectal cancer (CRC), pancreas cancers (PC), liver cancer (HCC), gastric cancer and esophageal cancer represent the second cause of cancer worldwide behind lung cancer[29]. In addition, biliary tract cancers as cholangiocarcinoma (CCH) also belonging to digestive cancers is less frequent. However, it should be noted that the incidence of PC which is mostly depicted (94%) by the pancreatic ductal adenocarcinoma (PDAC), is constantly increasing[31]. The factors increasing risk of digestive cancer include not only tobacco smoke, alcohol use, low physical activity, and diet, but another high-risk factor has also been identified, chronic inflammation such as IBD, pancreatitis, liver fibrosis and metabolic syndrome [32]. As mentioned above, OxA displays anti-inflammatory properties in IBD and other inflammatory diseases. These data indicate that orexin could play a role in triggering cancer. In 2004, our group tested the impact of 26 peptides including neuropeptides, hormones and orexins, on the cell growth of cancer cell line HT-29 derived from colorectal cancer[33]. The vast majority of these peptides had no significant effect on cell growth, only OxA and OxB inhibited the tumoral cell growth [33]. Analysis of this effect showed that orexins had no effect on cell cycle and cell proliferation but were able to induce a mitochondrial apoptosis in cancer cells[34,35]. Apoptosis was mediated by OX1R and OX2R however, only OX1R was ectopically expressed in human digestive cancers including CRC, PDAC, CHC and CCH[3,36]. Moreover, we observed that OX1R was also expressed in hepatic and lung metastasis from CRC[35]. It should be noted that OX1R and OX2R were not expressed in normal colon epithelium and in normal exocrine pancreas and liver[35,36]. However, as mentioned in the introduction, the main signaling pathway activated by orexin receptors involved the intracellular calcium release. Moreover, the inhibition of PLC enzyme which was activated by receptors in the presence of orexins *via* the Gq protein [37] was unable to inhibit the apoptosis process induced by orexins in cancer cells[33]. These observations indicated that the orexins/OX1R system triggered a new signaling pathway in cancer cells responsible of the pro-apoptotic peptide effect. Assessment of the new mechanism of action of orexins/OXR revealed that the interaction between orexins and their receptors induced phosphorylation of two immunoreceptor tyrosine-based inhibitory motifs (ITIM), present in the receptor sequences, induced by Src kinases[34,38]. Receptors phosphorylation led to the recruitment and the activation of tyrosine phosphatase SHP2 (Figure 2) followed by the activation of p38 mitogen-stress

protein kinase, translocation of Bax protein into the mitochondria, release of cytochrome c which participates to apoptosome formation and then activation of caspase 3 and 7 inducing DNA condensation and fragmentation causing the cancer cell death[34,38]. In preclinical mice models, we reported that when cancerous cell lines, such as HT-29 or LoVo or cells obtained from human colon tumors, were subcutaneously xenografted to mice administration of OxA and OxB were able to drastically reduced the tumor volume by apoptosis[35]. Although, preproorexin and OxA were immuno-detectable in total colon, no detection of preproorexin was obtained in normal and dysplastic epithelium[35] suggesting that endogenous OxA has no impact on tumoral growth. The colon cancer treatment was based mainly on surgery and chemotherapy but the primary cause of chemotherapy failure was associated to drug resistance[39]. Global studies indicated that more than 90% of patient cancer mortality was related to chemoresistance. The "gold-standard" treatment used in CRC was the 5-fluorouracil (5-FU). The development of HT-29 colon cancer cell line resistant to 5-FU demonstrated that OX1R was always expressed and orexins were able to induced pro-apoptotic effect in these cells suggesting that orexins response was conserved in drug-resistant cancer cells[35].

In PDAC, which represents the tenth most common cancer and the fifth in term of mortality[40,41], OX1R expression was detected in 96% of adenocarcinomas[36]. This expression was independent of the gender, patient age and tumor size[36]. OxA induced SHP2-dependent apoptosis in AsPC-1 cells derived from human PDAC as well as in PDAC slices from tumor patients maintained in culture[36]. The study using preclinical mouse models xenografted with AsPC-1 cells or cells isolated from patient's tumor indicated that OxA reduced the tumor development by induction of apoptosis [36]. OX1R was expressed at the early stage of development of digestive cancers including PDAC precancerous lesions named pancreatic intraepithelial neoplasia and dysplastic polyps in colon[3,36]. As mentioned above, various OxA antagonists such as almorexant or suvorexant have been developed for the treatment of insomnia. Surprisingly, AsPC-1 cells treatment with those antagonists showed that these two compounds inhibited PDAC cell growth by apoptosis induction[36]. Similarly, the intraperitoneal injection of almorexant in preclinical models led to inhibition of the tumor development indicating that this antagonist acted as OxA which was a full OX1R agonist[36]. These observations indicated that almorexant which blocked the intracellular calcium release induced by orexins was fully able to activate the pro-apoptotic signaling pathway in cancer cells. This type of molecule, able to discriminate various signaling pathways activated by one type of receptor, was termed biased ligand[42]. A very recent study on cryo-electron microscopy structure of OX2R active state revealed that one residue presents on the binding site play a central role in the receptor transition from the inactive to the active state[22]. This report could suggest that one or more residues in the receptor binding site drive the activation/inactivation of various signaling pathways. Moreover, this study confirmed the important role of Lys^{11} and Lys^{15} residues present in OxB, for the peptide interactions with its receptors [43]. OX1R was also expressed in HCC[44] and many studies reported that OxA had pro-apoptotic properties in gastric cancer[45], cholangiocarcinoma[3], esophagus cancer[35] but also in non-digestive cancer including prostate cancer[46] and neuroblastoma[35] in which apoptosis induced in these cancers by OxA was SHP2-dependent. Some report revealed that orexin receptors were also expressed in cortical adenomas[47], pheochromocytomas[48] and in endometrial carcinoma[49]. Observations indicated that OX1R was expressed in early stages of colon and pancreas cancer development[3,36] legitimately asking the following question: is the OxA/OX1R system is involved in chronic inflammation which may represent an important risk factor in tumorigenesis?

OREXINS AND IBD

The two major phenotypes of IBD were represented by ulcerative colitis (UC) and Crohn's disease (CD). These two disorders were characterized by a chronic inflammation of the intestine mucosa mediated by the immune system[50]. CD may affect any part of gastrointestinal tract from mouth to anus but often it concerns the part between the small intestine and the colon which can involve the deeper organ layer [51]. Whereas, UC affects mainly the colon and the rectum with a distal to the proximal gradient, characterized by superficial lesions having relapsing-remitting cycles[52]. These inflammations were characterized by persistent diarrhea, abdominal pain, bloody stools, great fatigue, immune cell infiltration and weight loss[52]. To date,

the etiology of these disorders remains unknown. IBD was associated to an increased risk (2-6 times) to develop CRC as compared to the general population[32,53]. Identification of UC pathogenic factors revealed that this disease involved numerous factors: genetic predisposition, epigenetic modifications, environmental factors including diet, geography, modern lifestyle, smoking, pollution, infections, exercise…, gut microbial factors (dysbiosis), inflammasome signaling pathways, endoplasmic reticulum stress and a disruption of immune response[54,55]. The treatment of UC was based on the severity and extension of the disease, involving the use of anti-inflammatory compounds such as 5-aminosalicylates, corticosteroids and immunosuppressive drugs such as azathioprine, 6-mercaptopurine, methotrexate, cyclosporine A, *etc*.[56]. The understanding of the role of various cytokines [Interleukin (IL)-6, IL-1β, tumor necrosis factor alpha (TNFα)] and other soluble mediators in UC, led to the development of therapies based on anti-TNFα, anti-IL-12/23, anti-integrin α4β7 and Janus kinase inhibitors[57]. The use of these drugs used alone or in combination has undoubtedly been a substantial advance in UC treatment in the last few decades. Unfortunately, important progress remains to be made to obtain curative treatment of IBD patients. The discovery of new targets is a main challenge for the therapeutic treatment of UC. In that respect, GPCR family represents a potential and innovative source of new targets. Several encouraging examples of GPCRs could play this therapeutic role in IBD through interaction with cannabinoid receptors[58], neuropeptide receptors[59], histamine receptors[60] and chemokine receptors[61]. Taking into account that OX1R was expressed in colon cancer and in precancerous lesions[10], the question is "Is OX1R expressed in IBD which represents a high risk to develop cancer?". Indeed, OX1R has been detected in inflamed mucosa from patient suffering of UC and CD[62]. It should be noted that OX1R was not expressed in normal human intestinal epithelium[35]. An epidemiologic study revealed that narcoleptic patients presented a higher prevalence to develop IBD[63]. To determine the role of OxA/OX1R system in ulcerative colitis, the classical DSS-induced colitis mouse model which reproduced the acute phase of colitis, was used. In this model, OX1R was ectopically expressed in inflamed mucosa[62]. Daily intraperitoneal injections of OxA revealed on this model, an anti-inflammatory effect of the peptide on the mucosa integrity and intestinal barrier[62]. OxA inhibited the secretion of various cytokines including IL-6, TNFα, IL-8, IL-1β, IL-1α, IL-17 and MCP-1 cytokines in intestinal mucosa and in immune cells extracted from colon[62]. In contrast, these anti-inflammatory effects were reverted by the SORA1 antagonist, SB-408124 demonstrating the specific effect of OxA which was fully mediated by OX1R *via* PLC signaling pathways (Figure 2)[62]. A recent report also showed that OxA was able to prevent the intestinal barrier disruption caused by lipopolysaccharide (LPS)[64]. To study the role of OxA/OX1R system on chronic inflammatory phases of UC, a genetically engineered mouse model invalidated for the IL-10 cytokine and NADPH Oxidase 1 was used. OxA peripheral injections induced the same anti-inflammatory effect compared to the DSS-induced colitis mouse model. This anti-inflammatory impact was mainly mediated by the activation of PLC that led to intracellular calcium release and inhibit Nuclear factor-κB (NF-κB) activation which plays a key role in pro-inflammatory cytokine secretion[65]. Even if only few studies have been devoted to the peripheral role of OxA in IBD, it seems clear that OxA/OX1R system displays anti-inflammatory properties in UC and may represent a promising new target in the treatment of this disease.

OREXINS AND SEPTIC SHOCK

Septic shock is a dramatic medical condition that represents a major health problem in response to a complex disorder arising from the dysregulation of an inflammatory response to infection that leads to low blood pressure and cellular metabolism abnormalities. Sepsis is caused not only by bacteria, but also by fungi, viruses or parasites. It could be located most frequently in the brain, lungs, urinary tract, skin or abdominal organs. It can lead to multiple organ dysfunction syndromes and death [66]. The pathophysiology of septic shock is not completely understood but an immune and coagulation response to the infection is the key role in the development of severe sepsis involving pro-inflammatory and anti-inflammatory responses. Septic shock was characterized by a widespread inflammatory response which produced a hypermetabolic effect. This effect was manifested by an increase of protein catabolism, cellular respiration and metabolic acidosis which was compensated by respiratory alkalosis[66]. LPS or endotoxins are major cell wall components of Gram-negative

bacteria, which induce systemic inflammatory response responsible of sepsis[67]. LPS-induced endotoxemia mouse model is one of the several well-studied animal models of septic shock[68,69]. Sepsis is characterized by an inflammatory cytokine secretion of TNFα, IL-6, IL-1β, and MCP-1[70]. NF-κB represents a therapeutic target since it induces pro-inflammatory gene transcription implicated in the septic shock[71]. In fact, in LPS-induced septic shock murine models, NF-κB inhibitors such as parthenolide and pyrrolidine dithiocarbamate[71], or an antagonist of toll-like receptor 4, the FP7[72] reverse sepsis effects on organ failure and hypotension. G protein-coupled receptors (GPCRs) may be potential targets for pharmacotherapy in sepsis. They could be involved in re-establishment of vascular endothelial barrier and alleviation of sepsis-induced organ dysfunctions. Several GPCRs and their associated ligands have been shown to play a role in septic shock but also in its treatment, including chemokine receptors (*i.e.*, ACKR2, CCR2, CCR5, CX3CR1, and CXCR1), neuropeptides (*i.e.*, VIP, neuropeptide Y, ghrelin, urotensin II, ocytocin, vasopressin, neurotensin, orexins, substance P, and apelin), proteases [*i.e.*, thrombin (PAR1 and PAR2)], lipid derivatives (*i.e.*, N-arachidonylglycine) and amines (*i.e.*, catecholamines, dopamine histamine, melatonin)[73]. Some reports indicate that intracerebral administration of orexin regulated body temperature and heart rate and increased the adrenocorticotropic hormone level in a mouse sepsis model induced by a caecal ligation associated to perforation[74]. The central administration of OxA to mice with endotoxin shock was shown to increase survival[75]. This report indicates that the exogenous administration of OxA was able to cross the blood barrier in systemic inflammation condition and induced an inhibition of IL-17, IFNγ, IL-6 and TNFα secretion[75]. Moreover, OxA restored body temperature and cardiovascular function in LPS-induced mice[75]. These authors hypothesized that OxA which was able to improve the survival of mice under septic shock condition, acts on the neuroendocrine and autonomic nervous system *via* the CNS, demonstrating a putative interest in treatment of septic shock.

OREXINS AND MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune dysregulation of the blood-brain barrier that affects the CNS through the migration of activated inflammatory cells. In the world, 2.3 million people are diagnosed with MS. The major causes listed for MS are inflammation, demyelination, oligodendrocyte loss, axonal loss and neurodegeneration. The neurodegeneration is a consequence of the inflammation induced by the demyelination which is related to the immune system activation[76]. T-cell mediated destruction of myelin and the autoimmune responses induced are still conjectural. However, this chronic inflammatory process involved a Th1/Th17 autoimmune response in the spinal cord and brain[77]. More recent data define MS as a primary degenerative disorder, which begins in oligodendrocytes that leads to neuroinflammation and to demyelination[78]. These neurodegenerative processes are present in different brain regions, including the hypothalamus and the orexinergic neurons that projected to various brain region encompassing hippocampus, cortical areas, striatum, and spinal cord[79]. As mentioned above, orexin neurons which represent about 70,000 neurons in the human brain, were involved in the regulation of sleep, pain, cognition, anxiety, alertness and motor function[80] and few studies proposed that orexin dysfunction could be connected to fatigue in MS[81]. Moreover, orexins have anti-inflammatory and neuroprotective properties by improving experimental autoimmune encephalomyelitis pathology[82]. In MS the T helper cell 1 cytokines are produced in large concentration by myelin activated T cells to activate microglial cells and macrophages. Then, they induce pro-inflammatory cytokines and chemokines, reactive oxygen, and reactive nitrogen species productions which are associated to demyelination and neurodegeneration. OxA inhibited reactive oxygen species production and also interleukin IL-1β, IL-6, and IL-8 expression. Similarly, to the inhibition of NF-κB activation by OxA in IBD[62], OxA was also able to reduce, in MS, the activation of NF-κB signaling pathways which leads to the expression of matrix metalloproteinase-3 (MMP3) and also to a MMP13 enzyme reduction in the tissue inflammation site[83]. Furthermore, OxA administration was shown to be able to cross the blood-brain barrier and reach the CNS in LPS-induced septic shock murine models [75].

Cerebral ischemia, and neurodegeneration induced by severe oxidative stress models, have been shown to be reduced by OxA. Moreover, OxA can active glucocorticoid secretion and the sympathetic nervous system through catecholamines releases

which had anti-inflammatory properties that reduces immune response[85]. In Parkinson's neurodegeneration disease, OxA had a neuroprotective impact[10] and in Alzheimer's disease, OXR activation also displayed a neuroprotective action[85]. Recently, the use of experimental autoimmune encephalomyelitis (EAE) model mimicking multiple sclerosis shown that orexins were able to induce anti-inflammatory effects. The intraventricular injections of OxA reversed clinical symptoms of EAE including partial or total paralysis of the two hind legs and death[82]. The authors also reported that treatment of the EAE mice model with OxA induced a large reduction in demyelination, microglial activation, and astrogliosis. OxA was shown to reduce nitric oxide synthase gene expression, an oxidative stress target which controls EAE pathology in the CNS. MMP9 (an inflammation enhancer) and IL-12 (a pro-inflammatory cytokine) expressions were also downregulated. Otherwise, OxA treatment limited $CD4^+$ T lymphocytes infiltration and Th1 and Th17 cytokines production induced by myelin oligodendrocyte glycoprotein (MOG35–55). OxA treatment also inhibited chemokine production as MCP-1/CCL2 and IP-10/CXCL10. Moreover, OxA reduced the cytokine production including IFN-γ, IL-17, TNF-α, IL-10, and TGFβ in the CNS[82]. Some of the common symptoms associated with MS are fatigue and sleep disturbances suggesting that MS and narcolepsy/catalepsy can share common genetic aspects[86]. Indeed, the physiological and psychological effects of MS are more severe in patients with sleep disorders[87]. To conclude, orexins which displayed immuno-modulating and neuroprotective properties reinforced by the orexinergic system involvement in the pathological development of multiple sclerosis become an interesting target as anti-inflammatory molecules for MS.

OREXINS AND METABOLIC SYNDROME

Control of Energy balance and metabolism is complex, they are monitored by the nervous and humoral systems. This section will consider the regulation of these functions by OxA and OxB or through their specific receptors. Orexins that are expressed in the lateral hypothalamus were first identified as factors that enhance feeding behavior. However, Hara *et al*[88,89] shown that orexin deficiency or postnatal ablation of orexin neurons induced mice obesity supporting that orexins are negative regulators of energy metabolism. Moreover, in human narcolepsy, related to orexin deficiency, is associated to a greater body mass index and to an increased risk of metabolic syndrome[90]. In contrast orexin overexpression protects rodents from diet-induced obesity and improves glucose control[91].

Orexin functions have been mainly described in the central nervous system but orexins and their receptors are also detected in various organs including the intestine, pancreas, adrenal glands, kidney, adipose tissue and reproductive tract. In peripheral tissues, orexins could affect insulin release, intestinal mobility, hormone secretion and blood pressure regulation[92]. Body weight and energy homeostasis are precisely controlled by many metabolic and hormonal factors including OxA. Orexins and their receptors have been located in the endocrine pancreas where they were co-located with insulin and beta cells[93] suggesting the role of orexin receptors in the glucose homeostasis. Some specificity of both isoforms of orexin and of each receptor in the control of energy balance have been reported but their roles remain unclear[92,94]. Recently plasma OxA have been negatively associated with insulin resistance and positively with insulin sensitivity in type 2 diabetes suggesting a functional role of orexin in the development of correlated obesity diseases[95]. These data support that orexin can modulate appetite, energy expenditure and glucose and lipid metabolism.

Orexin and food intake

Intra-cerebroventricular injections of OxA was shown to increase food intake in rats, while OxB was less effective[96]. However, orexin infusion stimulates feeding during the light period but not at the dark phase and induces no significant increase of the total food intake over 24 h[97]. In a previous study we reported that chronic treatment of mice under standard diet with daily intraperitoneal (i.p.) injections of OxA did not have any important effects on energy intake and energy expenditure, even if the i.p. OxA injections were sensed by the hypothalamus and affected the expression of several receptors and neurotransmitters in the hypothalamus[98]. Moreover, it has been proposed that orexin-induced feeding not simply follow the arousal state but increase the signal of low glucose and hunger[99].

Orexin and obesity

OxA deficiency is associated with narcolepsy and to higher risk of obesity suggesting that OxA deficiency can contribute to glucose homeostasis and insulin sensitivity. Transgenic mice in which orexin-containing neurons are ablated develop narcolepsy and obesity[88]. However, overexpression of OX2R in rats protects them from diet-induced obesity and improves glucose control and leptin sensitivity suggesting that triggering OX1R or OX2R did not regulate the same pathway (Figure 3)[91]. Moreover, lack of the orexins decreased energy expenditure and increase adiposity, principally through a reduction of physical activity. At the opposite exogenous OxA attenuates adiposity in rats and mice ingesting high fat diet. We also reported that i.p. injection of OxA to mice ingesting standard diet induced a small but significant reduction of visceral fat mass and adiposity but we did not observe any decrease of the subcutaneous fat, suggesting a lesser sensitivity of these fat pads to OxA[98]. These results support the potential anti-obesity effects of orexins. However, conflicting results have reported by different studies. *In vitro* studies using 3T3-L1 showed that OxA stimulates preadipocytes proliferation whereas OxB suppresses. Both OxA and OxB can stimulate pig adipocytes but no convincing evidence have been reported in humans[17,100]. It has also been shown that OxA contributes to changes of the white fat morphology *via* stimulation of preadipocytes proliferation and inhibition of apoptosis but OxA has no effect on lipolysis in fat tissue derived from human explants [101]. Moreover, OxA can raise corticosterone levels and glucocorticoids that can stimulate lipolysis in adipocytes[102]. In addition, *in vivo* studies reported that OxA potentiates physical activity and energy expenditure which reduce lipid accumulation.

Orexin and brown adipose tissue

Brown fat cell functions are different they generate heat. Many data support that OxA is required for adipogenesis of brown adipose tissue (BAT) in rodents. Activation of brown adipose tissue is controlled by environmental and hormonal factors as well as sympathetic neurons[103]. Ida *et al*[104] reported that cold stress stimulates the expression of preproorexin mRNA expression suggesting that orexin may be involved in body temperature control. Moreover, ablation of hypothalamic orexin neurons reduced BAT thermogenesis[105] and at the opposite central administration promotes thermogenesis[106]. A recent report, contrary to previous studies[107,108], did not show a direct effect of orexin on BAT development but a regulation of BAT by orexin signaling through the sympathetic system[109]. Moreover, *in vivo* OxA fail to stimulate the differentiation of human brown preadipocytes as well as the expression of the genes regulating thermogenesis.

Orexin and regulation of glucose homeostasis

Orexin deficiency has been associated with obesity, glucose intolerance and insulin resistance in rodents and humans[110]. OxA i.p. injections have been shown to increase GLUT4 expression in the liver suggesting that orexin can improve glucose uptake in hepatocytes, adipocytes and skeletal muscle[111]. Moreover, several studies show that orexin stimulates insulin secretion[112-114]. In rat model of type 2 diabetes mellitus (T2DM), an obesity treatment with OxA decreases fasting glucose and plasma levels of TNFα improve glucose control by increasing insulin sensitivity, increases plasma concentration of adiponectin and protects β-cells from apoptosis[114]. More studies are needed to better elucidate the mechanisms by which OxA modulates adipokines levels and other metabolic parameters such as the ability to reduce glucose. This effect can be a potential therapeutic approach for the treatment of DM and of its complications.

To summarize, a variety of data are presented in the literature. This disparity depends if the studies present *in vitro* or *in vivo* data and if the orexin administration was central (intraventricular) or peripheral. More studies will be needed to better define the mechanism by which the orexins regulate food intake, energy expenditure and glucose metabolism.

CONCLUSION

Since the identification of orexin peptides in hypothalamus demonstrating their crucial roles in sleep/wake regulation, the importance of their peripheral effects revealed their potential interests as therapeutic molecules in a wide range of human pathologies including also digestive diseases such as acute/chronic inflammation (IBD, septic shock, MS), metabolic syndrome and cancers.

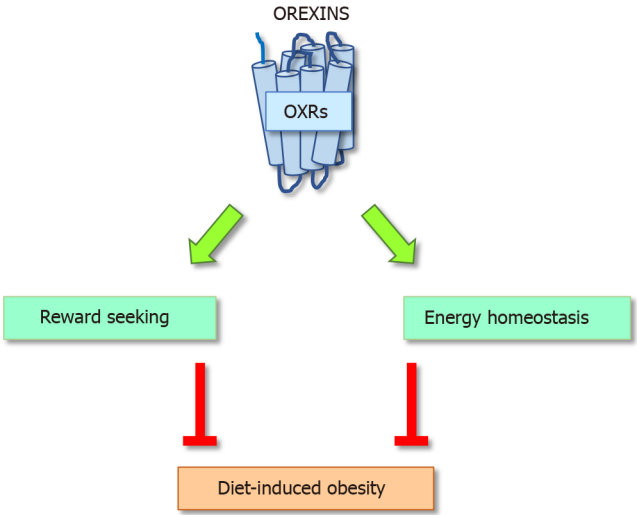

Figure 3 Main actions of orexins and their receptors on diet-induced obesity.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7597-7611

DOI: 10.3748/wjg.v27.i44.7597

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

OPINION REVIEW

Endoscopic management of difficult common bile duct stones: Where are we now? A comprehensive review

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Author contributions: Anderloni A conceived the idea for the manuscript; Tringali A and Costa D reviewed the literature and drafted the manuscript; Fugazza A, Colombo M, Khalaf K and Repici A reviewed the manuscript.

Conflict-of-interest statement: Alessandro Fugazza is a consultant of Boston Scientific and Olympus; Alessandro Repici is a consultant of Boston Scientific, Fujifilm and ERBE; Andrea Anderloni is a consultant of Boston Scientific, Olympus and Medtronic; No others conflict of interest for the remaining authors.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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Abstract

Endoscopic management for difficult common bile duct (CBD) stones still presents a challenge for several reasons, including anatomic anomalies, patients' individual conditions and stone features. In recent years, variable methods have emerged that have attributed to higher stone removal success rates, reduced cost and lower adverse events. In this review, we outline a stepwise approach in CBD stone management. As first line therapy, endoscopic sphincterotomy and large balloon dilation are recommended, due to a 30%-50% reduction of the use of mechanical lithotripsy. On the other hand, cholangioscopy-assisted lithotripsy has been increasingly reported as an effective and safe alternative technique to mechanical lithotripsy but remains to be reserved in special settings due to limited large-scale evidence. As discussed, findings suggest that management needs to be tailored to the patient's characteristics and anatomical conditions. Furthermore, we evaluate the management of CBD stones in various surgical altered anatomy (Billroth II, Roux-en-Y and Roux-en-Y gastric bypass). Moreover, we could conclude that cholangioscopy-assisted lithotripsy needs to be evaluated for primary use, rather than following a failed management option. In addition, we discuss the importance of dissecting other techniques, such as the primary use of interventional endoscopic ultrasound for the management of CBD stones when other techniques have failed. In conclusion, we recognize that endoscopic sphincterotomy and large balloon dilation, mechanical lithotripsy and intraductal lithotripsy substantiate an indication to the management of difficult CBD stones, but emerging techniques are in rapid evolution with encouraging results.

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

Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: July 23, 2021
Accepted: November 18, 2021
Article in press: November 18, 2021
Published online: November 28, 2021

P-Reviewer: Ozkan OF, Pelaez-Luna M
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

Key Words: Common bile duct stones; Balloon dilation; Endoscopic retrograde cholangiopancreatography; Endoscopic ultrasonography; Anastomoses, Roux en y; Double balloon enteroscopy; Mechanical lithotripsy; Cholangioscopy

Core Tip: The endoscopic management of difficult common bile (CBD) stones remains a challenge, whilst emerging techniques such as endoscopic sphincterotomy and large balloon dilation, mechanical lithotripsy and intraductal lithotripsy provide a procedural indication that align with the patient's condition, comorbidities, feature of the CBD stone and the patient's anatomical features. This review focuses on comprehensively outlining a stepwise approach for the management of difficult CBD stones and comparatively discusses indications depending on surgical altered anatomy and future indications in the management of difficult CBD stones.

Citation: Tringali A, Costa D, Fugazza A, Colombo M, Khalaf K, Repici A, Anderloni A. Endoscopic management of difficult common bile duct stones: Where are we now? A comprehensive review. *World J Gastroenterol* 2021; 27(44): 7597-7611
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7597.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7597

INTRODUCTION

About 10%-15% of biliary stone extraction procedures are demanding and require additional endoscopic techniques in order to allow stone clearance[1]. Factors that influence the technical difficulty of common bile duct (CBD) endoscopic clearance can be attributed to the patient's clinical condition, the stone's characteristics and anatomical factors (Table 1). Furthermore, the concomitant presence of Mirizzi syndrome and/or primary sclerosing cholangitis are also agreed upon by experts to make stone extraction a challenging procedure[2,3].

In accordance with the European Society of Gastrointestinal Endoscopy (ESGE) guidelines on endoscopic management of common bile duct stones, endoscopic sphincterotomy combined with endoscopic papillary large balloon dilation is considered the first line approach, reserving mechanical lithotripsy in case of failure [4]. Cholangioscopy-assisted lithotripsy has been increasingly reported as an effective and safe alternative technique to treat difficult CBD stones, even though its availability is still limited to referral centers[4,5]. Moreover, endoscopic management of CBD stones in patients with surgically altered anatomy (SAA) is technically demanding with a reduced rate of technical success[6]. Balloon assisted enteroscopy (BAE) has revealed to be effective in this setting, although its rate of failure has been reported to be up to 35%[7]. The use of interventional endoscopic ultrasonography (I-EUS) has been reserved to cases of BAE failure, due to its higher rate of adverse events when compared with BAE in previous reports[8-10]. Nevertheless, recent studies showed that I-EUS is efficacious with a low risk of adverse events, so it should be considered as first line treatment in expert hands and in referral centers[11]. The future of I-EUS requires the development of dedicated devices, making the procedure easier and safer with expanded indications[6,12]. Further studies will help to assess the role of I-EUS as the first approach for the management of CBD stones in patients with SAA. Finally, percutaneous cholangioscopy is also a novel alternative technique that should allow to treat difficult CBD stones in patients with SAA[13].

STEPWISE APPROACH FOR THE MANAGEMENT OF DIFFICULT COMMON BILE DUCT STONES

The first step is gaining access to the biliary tree, in order to remove the stone, which can be achieved by three different techniques: Endoscopic sphincterotomy (EST), endoscopic papillary large balloon dilation (EPLBD) and a combination of EST and

Table 1 Causes of difficult stone extraction

Category	Risk factors
Patient's clinical condition	Age > 65 yr; Bleeding tendency; Very poor medical condition
Stone characteristics	Stone size > 15 mm; Barrel or square shaped; Multiple stones > 3; Hard stone consistency; Intrahepatic/cystic duct location
Anatomical factors	Anatomical CBD factors: Narrowing of the bile duct distal to the stone, sigmoid-shape CBD, distal CBD angulation > 135°, short distal CBD < 36 mm; Periampullary diverticulum; Duodenal stricture; Surgically altered anatomy (Roux-en-Y gastric bypass or Billroth II with long afferent limb)

EPLBD [endoscopic sphincterotomy and large balloon dilation (ESLBD)]. Although the optimal choice remains debatable among endoscopists, the recently published ESGE guidelines[4] recommend ESLBD as the first-line approach to difficult CBD stones (in particular large stones), due to a 30%-50% reduction of the use of mechanical lithotripsy (ML) and a similar rate of technical success when compared to EST alone [14-20] (Figure 1).

However, balloon dilation is contraindicated in cases with distal biliary strictures, due to the increased risk of perforation[21], whereas EST increases the risk of bleeding in patients on antithrombotic agents[22]. A recently published systematic review and metanalysis including 13 randomized controlled trials conducted on 1990 patients[23] focused on the treatment of large CBD stones using the three techniques. Analyzing the surface under the cumulative ranking curve index, Lyu *et al*[23], concluded that EPLBD had the highest overall and initial success rates and the lowest probability of bleeding. ESLBD also had the lowest probabilities for the need for ML, risk of perforation, morbidity rates and risk of mortality. On the other hand, EST was associated with the lowest rates of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) and cholangitis. However, when each outcome was analyzed on pooled network analysis, no significant differences among the three groups emerged. There are many limitations in this study: The definition of the success rate and post-procedure complications varied in the included studies, the details of the different endoscopic procedures, such as the size of the dilation balloon, differed in the studies and the patient's characteristics changed among studies, such as bleeding predisposing factors and anatomical factors (*e.g.*, the presence of a peripapillary diverticulum and the size of the distal bile duct). To conclude, the technique for removing difficult CBD stones is still debated among endoscopists and needs to be tailored to patient's characteristics and anatomical conditions[24]. Higher numbers of high-quality trials are required.

When the size of CBD stones, even after balloon dilation, exceeds the diameter of the distal CBD, ML should be performed (Figure 2). The success of ML has been reported to range between 79% and 96%[25-29], with a low mortality rate and an overall adverse events (AEs) rate of 3.5% in a multicenter study[30], including trapped/broken basket, wire fracture and broken handle perforation/duct injury. Even though previous studies have focused on the importance of stone size as the factor that hampers stone clearance[29], Garg *et al*[28] demonstrated that the only predictor of unsuccessful ML was the stone impaction into the CBD. This determined the inability to push the basket proximal to the stone or the incapability to open fully the basket to grasp the stone.

ML can require multiple sessions to achieve stone clearance, undergo AEs and still be ineffective for difficult CBD stones[31]. In such cases, cholangioscopy-assisted intraluminal lithotripsy with electrohydraulic (EHL) or laser lithotripsy (LL) is the technique of choice to fragment large stones under direct visualization. There are three cholangioscopy techniques available: The oldest "dual-operator" mother-baby approach, the "single-operator" mother-baby approach (Spyglass, Boston Scientific, Natick, MA, United States) and the "direct" technique using currently available ultrathin gastroscopes[32].

Each of the three cholangioscopy technique allows EHL or LL under direct visualization (Figure 3). Nevertheless, in regards to which specific type of cholangioscopy and lithotripsy to use, it depends on local expertise and availability, as mentioned in ESGE guidelines[4]. There are currently no studies that compare cholangioscopy techniques. Concerning the type of lithotripsy used, Veld *et al*[33] recently published a systematic review comparing LL, EHL and extracorporeal shock wave lithotripsy (ESWL) in the treatment of difficult CBD stones after a previously failed ERCP. In their study, LL had a significantly higher complete ductal clearance rate (95.1%) compared

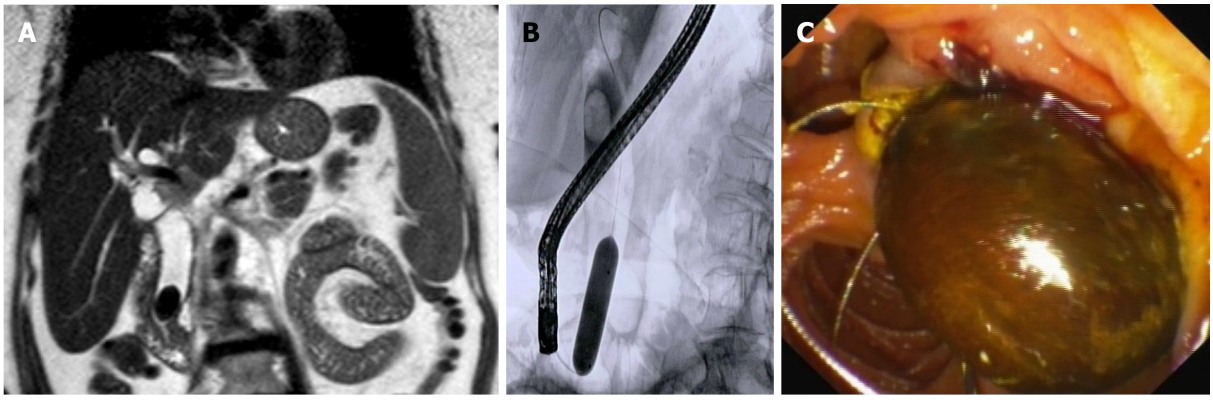

Figure 1 Management of difficult common bile duct stone by endoscopic sphincterotomy and large balloon dilation. A: Magnetic resonance imaging showing a large stone in the distal common bile duct; B: Fluoroscopic appearance of endoscopic papillary large balloon dilation with a pneumatic balloon filled with contrast medium; C: Final endoscopic view of the stone extracted by a Dormia basket.

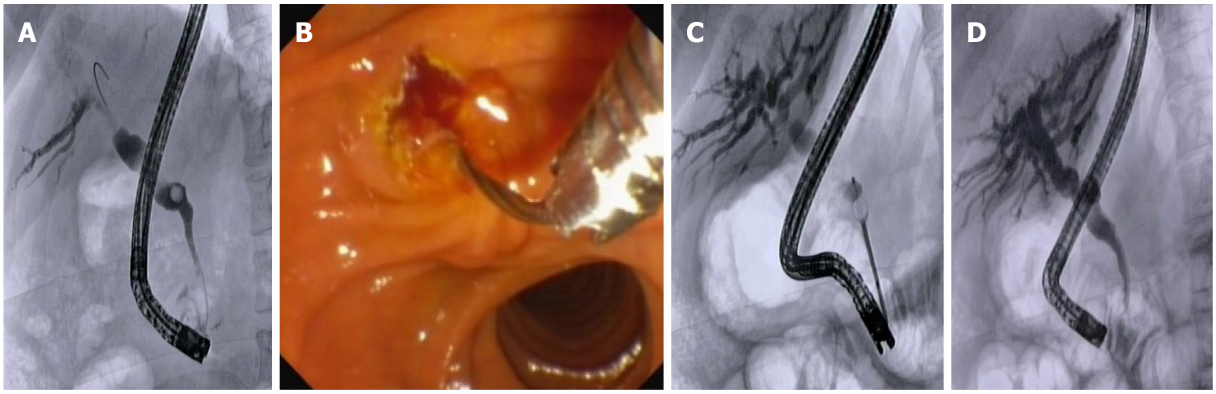

Figure 2 Management of common bile duct stones with distal biliary stricture by mechanical lithotripsy. A: Cholangiogram showing distal common bile duct (CBD) stricture with stone in the medium CBD; B: Introduction of a mechanical lithotripter over the Dormia basket; C: Mechanical lithotripsy under fluoroscopic control; D: Final cholangiogram showing complete CBD clearance.

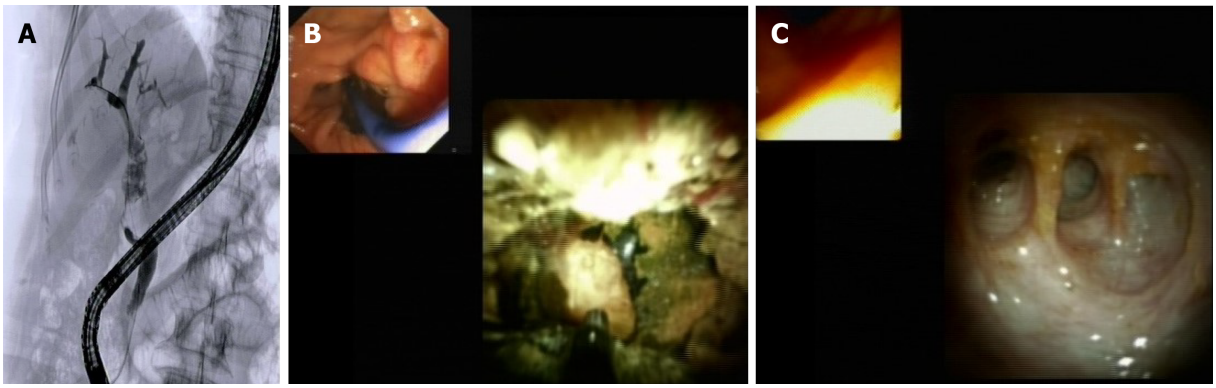

Figure 3 Management of impacted common bile duct stones with distal biliary stricture by cholangioscopy assisted lithotripsy. A: Cholangiogram showing distal common bile duct (CBD) stricture with large impacted stone in the medium CBD and multiple stones above; B: Cholangioscopy assisted lithotripsy by electrohydraulic of the impacted stone; C: Final cholangioscopy showing complete CBD clearance with biliary confluence appearance.

with EHL (88.4%) and ESWL (84.5%), while EHL had a higher post-procedural AEs rate (13.8%, including cholangitis, hemobilia and pancreatitis) compared with ESWL (8.4%) or LL (9.6%). In opposition to these results, a more recent meta-analysis[34] reported a superiority of EHL *vs* LL (mean successful endoscopic clearance rate 91.4% and 88.6%, respectively), explaining a more selective study inclusion than that made by Veld *et al*[33]. The last published meta-analysis comparing cholangioscopy-guided lithotripsy *vs* conventional therapy for complex bile duct stones[34] showed no

significant difference between ERCP and cholangioscopy in terms of therapeutic success, AEs rate and mean fluoroscopy time, but a shorter mean procedure time for conventional ERCP methods were found. In detail, cholangioscopy-guided lithotripsy showed a successful endoscopic clearance rate of 88.29% [95% confidence interval (CI): 86.9%-90.7%], first session successful endoscopic clearance rate of 72.7% (95%CI: 69.9%-75.3%), mean procedure time of 47.50 ± 6 min for session, number of sessions to clear bile duct of 1.5 ± 0.18 and adverse event rate of 8.7% (95%CI: 7%-10.9%). The majority of patients in the considered studies had a history of failure to remove stones on prior ERCP attempt. Therefore, Galetti *et al*[34] concluded that cholangioscopy-guided lithotripsy should be reserved to cases where the conventional techniques failed to achieve initially stone clearance. The majority of data published by Galetti *et al*[34] are in line with an older meta-analysis by Korrapati *et al*[35]. Regarding AEs, cholangitis is the most frequently reported, while PEP and perforation rarely occur [35].

Cholangioscopy-assisted lithotripsy should be reserved to selected cases and in the setting of tertiary care centers due to its costs, complexity and AEs rate. However, an increasingly number of authors claim that cholangioscopy-assisted lithotripsy may be considered first-line therapy for patients with difficult CBD stones in order to avoid serial procedures[36-38] and decrease costs[39]. Moreover, this procedure must be performed at tertiary centers by expert endoscopists.

When conventional ML fails and intraluminal lithotripsy is not available, the ESGE guidelines suggest to perform ESWL[4]. However, this procedure often requires multiple sessions, the placement of a naso-biliary drainage and subsequent ERCP to extract stone fragments. Ductal clearance rate appeared lower compared with LL (53%-73% *vs* 83%-97%, respectively)[40,41] but similar to EHL (78.5% *vs* 74%, respectively)[42]. ESWL-related AEs, including mostly cholangitis and pancreatitis, range from 9%-35.7%[40,42].

When biliary stones remain irretrievable but patients still need biliary drainage due to the risk of cholangitis, the placement of a temporary plastic stent is highly recommended before a second attempt at stone extraction can be made[4]. Horiuchi *et al*[43] documented that stent placement for 2 mo is associated with large and/or multiple CBD stones becoming smaller and/or disappearing without any complications, with a successful stone removal of 93% on a second ERCP[43]. Likewise, the disintegration of stones depends on the continuous friction between the plastic stent and the stones, which produces stress forces on the stone[43]. In a recent retrospective study, Jang *et al*[44] compared the use of different stents (7-Fr rather than 10-Fr plastic stents) in this setting, showing that the mean stone size reduction did not differ between the stents (5.7 mm in the 7-Fr stent group and 5.5 mm in the 10-Fr stent group; $P = 0.91$). However, when performing multivariate analyses, 7-Fr double pigtail stents significantly improved the complete clearance rate. On the other hand, the reduction in stone size was greater in the double-stenting group than in the single-stenting group, but the use of a double stent did not alter the complete stone clearance rate[44]. Covered self-expandable metal stent have been also used as an alternative to plastic stents to drain CBD after unsuccessful difficult stone removal. However, their high cost has not been certainly related to improved benefits[45-48].

DIFFERENT APPROACHES FOR PATIENTS WITH ALTERED ANATOMY

Endoscopic management in patients with SAA is still challenging for the endoscopists. Before performing ERCP, it is paramount to understand the anatomy and length of the afferent limb in order to select the appropriate approach, through the revision of the surgical report. The success of interventional endoscopy will depend on the correct choice of the endoscope and devices that should be tailored to the patient's anatomy [49].

Billroth II reconstruction

According to the ESGE guideline, a duodenoscope should be the first choice, reserving a forward-viewing endoscope (gastroscope, pediatric colonoscope, device assisted enteroscope) in case of failure[50]. Endoscopic sphincterotomy, where an inverted sphincterotome rather than precut following biliary stent placement in case of dedicated sphincterotome unavailability is used, is the standard of care in this setting. Furthermore, EPLBD could be used as an alternative method to sphincterotomy for CBD stones extraction, especially for stones larger than 10 mm[51,52]. Usually, the length of the afferent limb is short (less than 50 cm), but in cases of antecolic gastroje-

junostomy, it could be too long to be reachable by a duodenoscope. In this case, a forward viewing endoscope, allowing better visualization and easier intubation of the afferent limb compared with the lateral viewing endoscope, should be used. However, duodenoscope makes biliary cannulation easier using the elevator, even though it emerged to be associated with a higher perforation rate when compared with the forward viewing endoscope. This is due to limited visualization, difficult control of the scope, and the need to apply more pressure to overcome looping[53,54]. However, according to a recently published review by Krutsri[53], in patients with Billroth II gastrectomy, the duodenoscope has an afferent limb intubation success rate ranging from 62.5%-100%, cannulation success rate 88.2%-100% and complication rate 0%-12.5%. On the other hand, gastroscope with or without cap is reported to have similar results with an afferent loop intubation success rate of 76.8%-100%, cannulation success rate 81.4%-100% and complication rate 0%-10%. A subsequent retrospective study comparing sideviewing duodenoscope and forwardviewing endoscope to perform ERCP in patients with Billroth II gastrectomy reported afferent loop intubation rates of 95.1% for the side-viewing duodenoscope and 100% for the forward-viewing endoscope (P = 0.49). The rates of reaching the papilla were 70.7% and 91.1%, respectively (P = 0.06). Cannulation success rate after reaching the papilla was 100% in the side-viewing duodenoscope group and 90.3% in the forward-viewing endoscope group[55].

In 2015, Bove *et al*[56] reported a 30-year experience, showing that, in tertiary referral centers, patients with Billroth II (BII) that underwent ERCP had similar rates of morbidity and mortality when compared with patients with normal anatomy. In a recent systematic review and meta-analysis, Park *et al*[57] compared the efficacy and safety of forward viewing *vs* lateral viewing endoscopes and demonstrated that there was no statistically significant difference between the two endoscopes.

In referral centers, device-assisted enteroscopies (DAE) could be the first option because of higher technical success rate and lower adverse events, when compared with duodenoscopes and forward viewing endoscopes[53]. The majority of CBD stones in patients with Billroth II anatomy can still be removed by standard techniques such as EST and EPBD.

In a recent retrospective study, Duo *et al*[58] analyzed the risk factors for technical ERCP failure in Billroth II anatomy, demonstrating that in two or more CBD stones where the largest CBD stone measures to 12 mm or larger in size, stone characteristics for failed stone removal were included. Moreover, after the first ERCP attempt, Braun anastomosis and the use of no cap-assisted gastroscope were risk factors for technical failure of ERCP in this patients' cluster[58]. The initial stone removal rates of EPLBD have been reported to range from 66.7%-92.5%, while the overall stone clearance rates were from 96%-100%[51,59,60]. EPLBD, with or without EST, showed a high rate of first session stone clearance, reducing the need of endoscopic ML[51]. EPLBD has resulted to be associated with higher risk of post-ERCP pancreatitis in some studies [61-63], unlike most of the recent papers which showed the efficacy of EPLBD without increasing adverse events, including PEP[59,64]. On the other hand, EST is associated with a higher risk of bleeding[65]. However, sometimes lithotripsy is necessary to achieve stone clearance, especially when stones are too large to extract even after EPLBD, or when EPLBD is too risky in cases of distal bile duct narrowing or stricture [66].

There are three treatment options for lithotripsy: Endoscopic ML, cholangioscopic guided lithotripsy (LL or EHL) and ESWL. ML is a first treatment option, although it failed in cases where bile duct stones were larger than 2-3 cm, due to the difficulty to catch with a mechanical lithotriptor. In those cases, intraductal lithotripsy could be used, although cholangioscopy is difficult to carry out in patients with SAA. In these setting of patients, ESWL should be considered as a second option. However, endoscopic naso-biliary drainage is necessary before performing ESWL, which has a lower efficacy compared to LL or EHL[40].

Finally, EUS-guided intraductal lithotripsy or percutaneous transhepatic biliary drainage should be considered if ESWL is ineffective. In the percutaneous transhepatic biliary drainage approach, CBD stones are extracted in the antegrade fashion after balloon dilation of the papilla. In cases with large CBD stones, percutaneous transhepatic cholangioscopy with intraductal lithotripsy or ESWL could be attempted to facilitate stone removal. EUS-guided approach has gained popularity in the management of bile duct stones in patients with BII anatomy. In a retrospective study [9], EUS-guided antegrade (EUS-AG) treatment had a technical success rate of 72%, due to the technical difficulty of the antegrade stone extraction. Moreover, mechanical or intraductal lithotripsy, through an enterobiliary fistula after fistula maturation, could be made[67].

Roux-en-Y reconstruction

Roux en Y reconstruction can be divided in Roux en Y without gastric bypass (*e.g.*, Roux en Y gastrojejunostomy, Roux en Y hepaticojejunostomy, pancreatico-duodenectomy) and with gastric bypass (RYGB). Roux-en-Y reconstruction, compared to BII surgery, results in a longer and tortuous limb, increasing the difficulty to reach the papilla. In this setting, patients should be managed in referral centers where DAEs are the first option[68], with a reported cannulation rate ranging from 58%-95.6%, and an AEs rate between 7% and 10%, with a perforation rate of 0%-3.2%[69,70].

A systematic review and meta-analysis showed that BAE has a high diagnostic and procedural success rate in patients with Roux-en-Y reconstruction (69.4% and 61.7%, respectively), with an overall AEs rate of 6.5%[71]. Different studies have shown that there is no superiority among different DAE methods [single balloon enteroscopy (SBE), double balloon enteroscopy (DBE) and spiral enteroscopy][72]. Nevertheless, three systematic review and meta-analyses demonstrated better results for DBE compared to SBE (the success rate of reaching the papilla and treatment were 89.7% and 63.5% for DBE and 80.9% and 61.7% for SBE, respectively)[71,73,74].

The success rate of forward viewing endoscopes could be increased by using an underwater cap assisted technique, which combines the use of a cap applied to the tip of a pediatric colonoscope, with the injection of water as an alternative medium to carbon dioxide or air to distend the bowel lumen. It appeared that on one side the underwater technique reduces loop formation and bowel distension, while on the other side, the use of a cap improves the visualization of the papilla and helps in maintaining a stable position[75].

Even after cannulation, extraction of CBD stones can be difficult in patient with Roux-en-Y reconstruction, although different techniques have been described in this cluster of patients. EPLBD has been reported to achieve complete stone removal on a single-session in 66.7%-100%, while overall complete stone removal was obtained in 96.7%-100% of cases[49]. ML can be used, even though it is often technically challenging during enteroscopy-assisted ERCP and may fail.

Therefore, direct peroral cholangioscopy, through direct insertion of an ultra-slim endoscope or an enteroscope into the bile duct, allowing to perform lithotripsy, has been described in some case reports[76-80]. Some authors have also described the placement of an overtube through the scope, which allows the insertion of a cholangioscope (Spyglass, Boston Scientific) and direct lithotripsy[81,82].

EUS-AG stone treatment has also gained popularity. Biliary access is achieved from the stomach or jejunum under EUS-guidance, using a guidewire that is passed through the ampulla into the duodenum. Then the ampulla is dilated using a balloon, and finally, CBD stones are pushed into the duodenum using a stone extraction balloon.

The reason for technical failure is the failed puncture of the intrahepatic bile duct, guidewire passage and difficulty to stone extraction due to large stones size. However, these reasons could be overcome using large balloon dilation. Nevertheless, the maximum balloon size is limited to the size of the distal CBD, therefore intraductal lithotripsy is needed in cases with stones larger than the size of the distal CBD, increasing the risk of bile leak. However, a two-step approach has been proposed to prevent bile leaks and allowing safe usage of ML and cholangioscopy in EUS-AG stone treatment[67].

Mechanical lithotriptor can be introduced over the guidewire and into the bile duct, through the fistula. A fistula dilation up to 10-F using a plastic stent, a fully covered self-expandable metal stent after endoscopic ultrasound-guided hepaticogastrostomy or endoscopic ultrasound-guided hepaticojejunostomy, should be made in order to prevent bile leak and to allow easy access of the cholangioscope into the biliary system [83].

For the management of complex CBD stones, with the use of DAEs in patients with SAA has been studied in larger cohort of patients, EUS-guided therapy in this setting has been increasingly reported in case reports[8-11,84-86]. There are advantages and disadvantages in enteroscope-assisted stone management and EUS-guided interventions in patients with Roux-en-Y anatomy.

Enteroscopy-assisted ERCP uses the physiological biliary access and has a lower risk of bile duct leakage, although scope insertion can be challenging. On the other hand, EUS-guided approach involving the puncture of the left intrahepatic bile duct has a lower risk of bile leak but can be challenging when the intrahepatic bile duct is minimally dilated. Enteroscopy-assisted ERCP should be the first approach, reserving EUS-guided approach in case of failure as a salvage technique. We should keep in mind that enteroscopy-assisted ERCP and EUS-AG stone treatment need expertise as well as dedicated devices, therefore these procedures should be performed by skilled endoscopists in high volume referral centers.

Roux-en-Y gastric bypass

The treatment of CBD stones in patients who underwent weight loss surgery, especially RYGB, is challenging due to difficult access to the CBD. In fact, in Roux en-Y reconstruction, the afferent limb length can be more than 200 cm, with sharp angulation of the jejunojejunal anastomosis, severe adhesion and looping of the scope. Moreover, the incidence of symptomatic gallbladder disease is around 15% after significant weight loss, therefore prophylactic cholecystectomy has been suggested [87]. The percentage of therapeutic success of ERCP using a pediatric colonoscope or with DAEs has been reported around 60%[88]. New techniques to perform ERCP in this setting are EUS-directed transgastric ERCP (EDGE) and laparoscopic-assisted transgastric ERCP (LA-ERCP), which reach a success rates of 80%-100%[89-91]. However, both procedures have some limits. EDGE requires expertise in interventional EUS and ERCP, has higher costs and can be associated with stent migration and subsequent perforation[92,93] and permanent gastro-gastric fistula with weight regain [94]. On the other hand, LA-ERCP needs coordination between surgeon and endoscopist with a gastrostomy tube left *in situ*, if multiple ERCP procedures are required to obtain stones clearance[95].

EDGE is a two-step procedure; procedures can be performed in the same session or in two separate sessions (Figure 4). Single session EDGE is associated with a higher risk of perforation due to lumen apposing metal stent dislodgement, while dual session has a lower perforation risk, but it requires 10-14 d interval to allow fistula maturation. A shortened interval dual session (2-4 d) has been proposed to overcome this limitation, decreasing the risk of intraprocedural 20 mm lumen apposing metal stent dislodgement and allowing a timely transgastric ERCP[96].

An international, multicenter trial comparing EUS-guided gastro-gastrostomy-assisted ERCP *vs* enteroscopy-assisted ERCP (e-ERCP) in patients with RYGB has shown that EUS-guided gastro-gastrostomy-assisted ERCP may be superior to e-ERCP in terms of higher technical success and shorter procedural time, with similar safety profile[97]. LA-ERCP provides the opportunity to perform cholecystectomy concomitantly with CBD stones clearance, in case the gallbladder is still in place[98].

Post-liver transplantation

CBD stones after liver transplantation have an incidence between 4% and 10% of cases [99]. Biliary strictures are the major predisposing risk factor for biliary CBD stones, occurring in up to 90% of liver transplant patients with biliary stones[100]. Other possible agents for stone formation are cold ischemia, hyperlipidemia, hypercholesterolemia, infections and cyclosporine assumption[100,101]. Biliary stones in post-liver transplantation patients may cause severe complications such as pancreatitis, biliary infections and biliary cirrhosis, which can drastically worsen the graft's course. The endoscopic management of CBD stones in this setting of patients is successful in over 90% of cases, although the presence of anastomotic strictures results in a challenging and demanding procedure and increases the difficulty of stones removal[99]. In this context the strictures have to be treated simultaneously with stone extraction, using balloon dilation and mechanical lithotripsy and reserving the use of cholangioscopy-assisted lithotripsy in case of failure[102]. CBD stones may be mistaken with biliary casts, which occur in 2.5%-18% of post-liver transplant patients due to hepatic ischemic injury and are associated with poorer graft survival[103]. The differential diagnosis is mainly based on cholangiography features in addition to the endoscopist experience and can be confirmed by cholangioscopy direct visualization. This is fundamental to direct the treatment approach.

FUTURE RESEARCH DIRECTIONS IN THE MANAGEMENT OF DIFFICULT CBDS

The use of cholangioscopy-assisted lithotripsy in patients with difficult CBD stones as a first step rather than after failed traditional treatment, need to be assessed in randomized controlled trials. Many endoscopic techniques have been used in patients with difficult CBD stones and altered anatomy, with variable results. Percutaneous transhepatic cholangioscopy, which has been recently reported as a novel and alternative approach for patients with SAA, allows access to the biliary tree and stones fragmentation under direct visualization[13,104,105]. However, efficacy and safety of its use need to be further investigated.

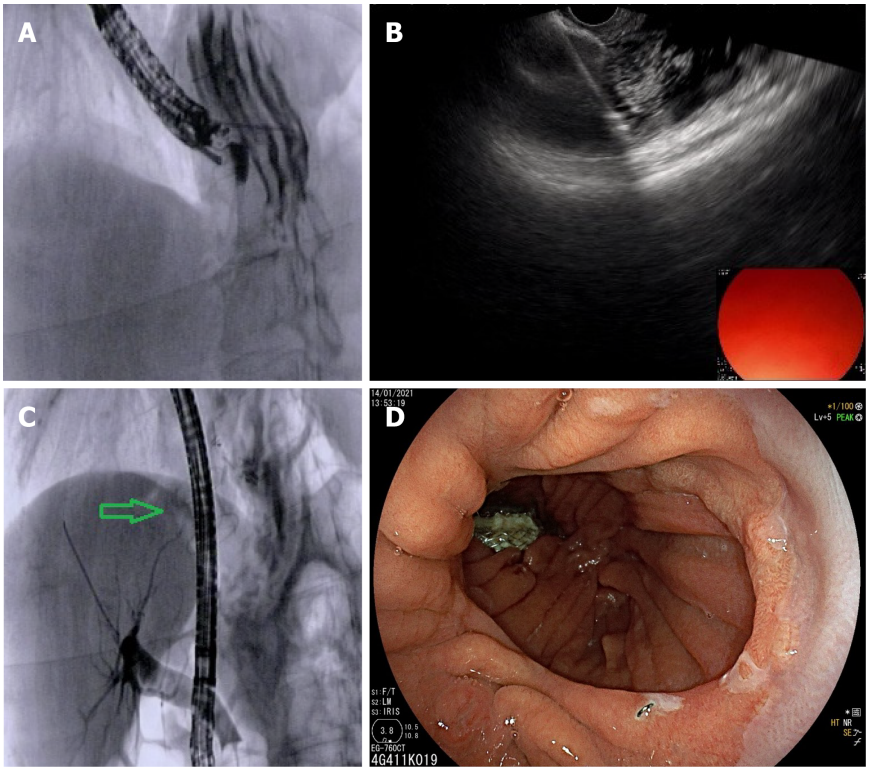

Figure 4 Endoscopic ultrasonography-directed transgastric endoscopic retrograde cholangiopancreatography for management of common bile duct stone in patient with previous Roux-en-Y gastric bypass for bariatric surgery. A: Endoscopic ultrasonography (EUS)-guided puncture of the excluded stomach with a 19G EUS needle with injection of contrast medium and sterile saline for gastric distension under fluoroscopic control; B: EUS guided first flange deployment of 20 mm lumen apposing metal stent (LAMS) into the gastric remnant; C: Endoscopic retrograde cholangiopancreatography for stone removal was performed after advancing the duodenoscope through the LAMS (green arrow); D: Endoscopic image confirming placement of the LAMS within the gastric pouch.

EUS guided interventions for biliary drainage after failed ERCP are in rapid evolution[106-107], including management of CBD stones in patients with normal anatomy when other techniques have failed[108]. Two retrospective studies and a recent review have compared EUS-rendezvous with precut papillotomy technique, showing that treatment success was significantly higher for EUS-rendezvous than for those with precut papillotomy, without significant differences in terms of complication rate[109-111]. Therefore, in tertiary referral centers, EUS-rendezvous could be used instead of precut papillotomy. The role of EUS-guided biliary treatment in patients with SAA should be investigated as primary technique in well-designed studies comparing safety and efficacy of EUS-guided interventions with enteroscopy-assisted ERCP. Finally, regarding patients with RYGB, more studies are needed in order to assess the role of the three different methods used in this setting (EDGE, LA-ERCP, e-ERCP), allowing endoscopists to tailor the technique to the patient.

CONCLUSION

The step-up approach involving ESLBD, ML and intraductal lithotripsy for patients with difficult CBD stones has been quite well validated. On the other hand, many different techniques involving the use of EUS in this setting are in rapid evolution with encouraging results. In patients with SAA, the endoscopic management of CBD stones is still challenging and should be managed in referral centers. The role of EDGE compared with LA-ERCP, e-ERCP as well as the efficacy and safety of percutaneous transhepatic cholangioscopy need to be evaluated in further well-designed studies. Finally, the definition of difficult CBD stones includes many different clinical scenarios with distinctive outcomes depending on the treatment choice. The correct pre-operative evaluation of the patient could help in choosing the best treatment strategy, in order to avoid unnecessary, ineffective ERCP session/attempt and can offer the best therapeutical approach to our patients.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7612-7624

DOI: 10.3748/wjg.v27.i44.7612

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

EVIDENCE REVIEW

Role of early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: An update of the evidence and future directions

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Author contributions: Khan F performed research and wrote the manuscript; Tripathi D contributed to critical revision of the manuscript for important intellectual content.

Conflict-of-interest statement: Khan F has no conflict of interest. Tripathi D receives Speaker fees and consultancy for Gore Medical, and Research grant award for early TIPSS trial (NIHR, UK).

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and Hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

Variceal bleeding is a serious complication of cirrhosis and portal hypertension. Despite the improvement in management of acute variceal bleed (AVB), it still carries significant mortality. Portal pressure is the main driver of variceal bleeding and also a main predictor of decompensation. Reduction in portal pressure has been the mainstay of management of variceal bleeding. Transjugular intrahepatic porto-systemic stent shunt (TIPSS) is a very effective modality in reducing the portal hypertension and thereby, controlling portal hypertensive bleeding. However, its use in refractory bleeding (rescue/salvage TIPSS) is still associated with high mortality. "Early" use of TIPSS as a "pre-emptive strategy" in patients with AVB at high risk of failure of treatment has shown to be superior to standard treatment in several studies. While patients with Child C cirrhosis (up to 13 points) clearly benefit from early-TIPSS strategy, it's role in less severe liver disease (Child B) and more severe disease (Child C > 13 points) remains less clear. Moreover, standard of care has improved in the last decade leading to improved 1-year survival in high-risk patients with AVB as compared to earlier "early" TIPSS studies. Lastly in the real world, only a minority of patients with AVB fulfil the stringent criteria for early TIPSS. Therefore, there is unmet need to explore role of early TIPSS in management of AVB in well-designed prospective studies.

Received: April 19, 2021
Peer-review started: April 19, 2021
First decision: May 12, 2021
Revised: May 27, 2021
Accepted: October 29, 2021
Article in press: October 29, 2021
Published online: November 28, 2021

P-Reviewer: Debnath P
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

In this review, we have appraised the role of early TIPSS, patient selection and discussed future directions in the management of patients with AVB.

Key Words: Transjugular intrahepatic portosystemic stent-shunt; Early transjugular intrahepatic portosystemic stent-shunt; Salvage transjugular intrahepatic portosystemic stent-shunt; Portal hypertension; Acute variceal bleed; Hepatic encephalopathy

Core Tip: Outcome of high-risk patients following episode of acute variceal bleeding (AVB) is poor and insertion of transjugular intrahepatic portosystemic stent-shunt (TIPSS) within 72 h of index endoscopy (early or pre-emptive TIPSS) is associated with remarkable outcomes in a selection of patients (Child C up to 13 points). However, it's efficacy in Child B patients is debatable and criteria for high-risk patients needs to be refined. Moreover, management of variceal bleeding has improved in last decade and provision of early TIPSS (within 72 h) is challenging in most healthcare facilities. In this paper we have discussed the role of early TIPSS, patient selection and future directions in management of AVB.

Citation: Khan F, Tripathi D. Role of early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: An update of the evidence and future directions. *World J Gastroenterol* 2021; 27(44): 7612-7624
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7612.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7612

INTRODUCTION

Acute variceal bleeding (AVB) is a severe complication of portal hypertension and occurs at a rate of around 10%-15% per year in patients with cirrhosis. The risk of variceal bleeding depends on the severity of liver disease, size of varices, and presence of red wale marks[1]. Six-week mortality following an episode of AVB (the endpoint identified as the key outcome in variceal bleeding) is reported to be between 15% and 25%[2-4]. Early mortality was reported to be 50% in the early eighties[5]. The presence of clinically significant portal hypertension is the main factor determining the risk of development of varices and other liver-related decompensations. Transjugular intrahepatic portosystemic stent-shunt (TIPSS) was initially used for management of refractory variceal bleeding only (salvage or rescue TIPSS), followed by prevention of rebleeding or as secondary prophylaxis. There has been recent interest in early or pre-emptive TIPSS (e-TIPSS) in selected patients at high risk of treatment failure and mortality. There remains considerable controversy in the utility of early TIPSS, and we aim to provide a summary of the current evidence and discuss unresolved issues and future directions.

IDENTIFYING PATIENTS AT RISK OF A POOR OUTCOME FOLLOWING AVB

Although the prognosis of AVB has significantly improved over the last decades due to better management of haemorrhage and its associated complications, mortality is still as high as 15%-20%[2].

Patients presenting with AVB do not benefit equally from standard treatment as not all patients have the same risk profile of treatment failure, re-bleeding and mortality. The risk of rebleeding (and subsequently death) is greatest in the first 48-72 h after the initial episode and over 50% of rebleeding episodes occur within the first 10 d[6-8]. Therefore, it is important to identify those patients who are at high risk of treatment failure and death in whom a more aggressive approach, like implantation of early or pre-emptive TIPSS (within 72 h of index bleeding) can be utilised.

Measurement of the hepatic venous pressure gradient (HVPG) is the gold standard method for evaluating portal hypertension[9]. Portal hypertension is defined as an increase of HVPG > 5 mmHg; and HVPG ≥ 10 mmHg is defined as clinically significant portal hypertension as above this threshold, varices usually appear and risk of developing overt clinical decompensation (variceal bleeding, ascites and hepatic encephalopathy) increases[9,10]. If varices remain untreated, rebleeding and death occur in approximately 60% and 30% of patients respectively, one to two years after the index bleeding[1].

HVPG measured within 24 h of the bleeding episode is shown to be a prognostic indicator for outcome following AVB. HVPG > 20 mmHg has been associated with up to 5-fold increased risk of failure to control bleeding and one-year mortality[11,12]. Decrease in portal pressure of ≥ 20% from the baseline or to HVPG ≤ 12 mmHg is associated with significant reduction in risk of decompensation and with improved survival[13].

Recent data also show that decreasing HVPG by > 10% from baseline, or to absolute values < 10 mmHg, reduces the risk of development of varices and AVB regardless of the presence of varices[14]. Therefore, lowering HVPG has been one of the treatment strategies in management of AVB.

Portal hypertension correlates strongly with severity of liver disease measured by Child-Pugh score[13]. The severity of liver disease remains the main determinant of prognosis in patients with AVB[15,16]; There is a strong relationship between the presence of HVPG > 20 mmHg and Child–Pugh class[11,17]. Therefore, Child-Pugh Class C is associated with poor outcome following AVB. Moreover, presence of ascites and bacterial infections are also associated with poor outcome[18].

Severity of bleeding (active bleeding on endoscopy and haematocrit level) as well as presence of portal vein thrombosis are also among the significant predictors of early treatment failure following AVB[19].

Recalibrated MELD score (−5.312 + 0.207 × MELD) has been developed to predict early mortality after an episode of AVB. MELD score of 19 or higher is associated with a higher mortality risk of 20%[2]. The utility of recalibrated MELD in predicting outcome has recently been validated in 2 observational studies[20,21]. Similarly, Child-Pugh Class C is associated with higher mortality risk than in Child–Pugh class A and B cirrhosis, regardless of the presence of active bleeding[21].

In a recently published study acute-on-chronic liver failure (ACLF) at baseline is also found to be an independent risk factor for rebleeding and mortality in patients presenting with AVB. Presence of ACLF almost doubled the risk of rebleeding[22].

SALVAGE TIPSS

In the 1980s, the prognosis in patients with refractory or uncontrolled variceal bleeding was poor with mortality of over 90% in Child-Pugh B and C patients[23]. Though rescue surgical treatments (oesophageal transection or surgical porto-systemic shunting) were effective in decreasing portal hypertension, these procedures were associated with high mortality, ranging from 50% to 90% in this situation[24,25]. Moreover, subsequent liver transplantation may become technically more difficult to perform following porto-systemic shunt surgery[25].

The concept of percutaneous transjugular porto-systemic shunt in context of oesophageal variceal bleeding in humans was first introduced by Colapinto *et al*[26] in 1982 (in which intrahepatic portosystemic shunt was created by dilating the track with an angioplasty balloon). First (prospective) study evaluating the role of salvage TIPSS in patients with variceal haemorrhage refractory to (then) standard medical and endoscopic treatment was published in 1994[27]. In that study though salvage TIPSS (with bare stent) was associated with immediate control of bleeding in all 20 patients, 40-d mortality was very high at 60% mainly due to liver failure and sepsis[27].

Several (retrospective) studies were published afterwards, evaluating the role of salvage (rescue) TIPSS (using uncovered stents) in setting of refractory variceal bleeding[28-30]. Salvage TIPSS was effective in controlling the variceal bleeding but early mortality rate remained high in these patients, approaching 48% at 45-d. Majority of the patients died due to multi-organ failure and sepsis. Child-Pugh (CP > 11), APACHE II and MELD scores (> 20) were associated with increased mortality[29,30]. These studies were uncontrolled, mainly involved uncovered stents and sclerotherapy was the choice of endoscopic treatment.

Standard treatment of AVB has improved considerably in the recent decade and covered TIPSS has lower risk of stent dysfunction as compared to bare metal stents[31,

32]. In subsequently reported retrospective studies of salvage TIPSS using both covered and uncovered stents, the use of covered stent did not culminate in survival advantage at both 6 wk and 1-year[33,34]. However, use of bare metal stent was associated with increased rate of re-bleeding due to stent dysfunction and salvage TIPSS appeared to be futile in patients with Child-Pugh score of > 13[34].

A recently published Chinese retrospective study of 58 patients, in which 55 patients had covered stents, showed better 6-weeks and 1-year transplant free survivals (87.7% and 81.8%, respectively) following salvage TIPSS[35]. Treatment failure at 6 wk was associated with bare stents and white cell count. It is important to note that 62% patients had Child B disease and over 60% had hepatitis B related disease. Only 30% of patients had Child C disease. Median MELD score was 10 and mean Child score was 8.7, indicating that majority of patients had less severe disease (but with high portal pressure)[35]. Moreover, 82% of patients had variceal embolization[35], an effective tool to prevent re-bleeding[36,37].

EARLY TIPSS

Randomised control trials in e-TIPSS

It is important to clarify the concept of e-TIPSS. e-TIPSS strategy refers to a pre-emptive placement of TIPSS in those at a high-risk of treatment failure before treatment failure or re-bleeding occurs. In this setting, TIPSS is usually placed within 24–72 h of successful therapeutic endoscopy (with patients already on pharmacological therapy with vasoactive drugs and antibiotics). The rationale of this strategy is that reducing portal pressure early on, will prevent rebleeding, the associated liver failure and development of multi-organ failure with a lot worse outcome. This is in contrast to salvage TIPSS, where TIPSS is placed in patients with refractory variceal bleeding, not controlled with standard treatment; and this group of patients has very high mortality (as described above).

As stated earlier, reduction in portal hypertension is one of the mainstays of management of AVB. Utilising this evidence, Jalan *et al*[38] introduced the concept of preventive insertion of TIPSS (pre-emptive or early TIPSS placement, within 72 h) to lower portal pressure in cirrhotic patients with AVB in 1990s. They published a randomised control trial (RCT) in 1997 including 58 patients and compared endoscopic band ligation (EBL) with e-TIPSS (with bare-metal stent) randomised within 24 h after controlling of first episode of AVB. Mean time to TIPSS in that study was 2.2 d. e-TIPSS placement was superior to EBL in preventing rebleeding and was cost-effective in this setting. However, no survival difference was seen, although ITU requirement was significantly less with TIPSS. The Child-Pugh score of 9 was similar in the two groups, although there were some Child's A patients included[38]. Patient selection was not as strict as for subsequent studies. This could explain the lack of difference of survival.

Since then, the role of e-TIPSS in the management of acute variceal bleeding in patients with cirrhosis has been evaluated in several studies. The safety and efficacy of e-TIPSS in high-risk cirrhotic patients has been evaluated in a few RCTs (Table 1).

Monescillo *et al*[12] performed the first study applying high-risk selection criteria by using measurements of HVPG. Fifty two patients with HVPG ≥ 20 mmHg measured with 24 h of bleeding episodes were randomised to either TIPSS group or standard therapy. Their study showed that "early" TIPSS placement was associated with a significantly lower rate of treatment failure (50% *vs* 12%) and lower 6-wk mortality (38% *vs* 19%). 46% of study population had Child C disease[12]. However, bare-metal stents were used in TIPSS patients and standard of care (SOC) in the non-TIPSS group did not reflect current practice (sclerotherapy rather than combination of endoscopic band ligation and non-selective beta-blocker therapy). Patients in non-TIPSS arm received only non-selective beta-blockers (NSBBs) to prevent rebleeding and EBL was used in whom NSBBs were not tolerated or were contraindicated.

Measurement of early HVPG for risk stratification and treatment assignment in AVB is not easily applicable in clinical practice nor readily available. Therefore, it is important to identify non-invasive predictors of treatment failure and early mortality in patients with AVB. In this regard, Abraldes *et al*[11] not only showed a strong relationship between the presence of HVPG > 20 mmHg and Child-Pugh class C but also showed that 6-wk mortality is more strongly determined by the severity of underlying liver disease (assessed by Child- Pugh classification) than by HVPG > 20 mmHg. Therefore, subsequent studies used clinical criteria to define high-risk patients and used only covered stents. A schema of the study design of these trials is illustrated

Table 1 Early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: Key studies

Ref.	Main inclusion criteria	Primary and secondary outcomes	Results	Comments
Randomised controlled trials				
Monescillo *et al* [12], 2004 (Italy)	HVPG > 20 mmHg within 24 h of admission.	(1) Primary: Sensitivity and specificity of HVPG cut-off value (20 mmHg) in predicting transplant-free survival (TFS), and assessment of TFS as well as short- and long-term survival; and (2) Secondary: Transfusional needs, ICU stay, complications during the first week of treatment, and causes of death.	6-wk mortality = 17% in e-TIPSS *vs* 38% in control ($P \leq 0.05$). 1-yr mortality = 31% in e-TIPSS *vs* 65% in control ($P \leq 0.05$). Treatment failure = 12% in e-TIPSS *vs* 50% in control ($P = 0.001$).	46% of study population had Child C and 40% had Child B cirrhosis. mean Child score = 9.2. SOC does not reflect current management and only bare metal stents were used.
García-Pagán *et al*[39], 2010 (Europe)	Child- B with active bleeding or Child C < 14 points.	(1) Composite Primary: Failure to control bleeding and failure to prevent clinically significant VB within 1 yr; and (2) Secondary: Mortality at 6 wk and at 1 yr, failure to control acute bleeding, early rebleeding, rate of rebleeding between 6 wk and 1 yr, other complications of PHTN, number of days in ICU, days spent in the hospital, use of alternative treatments.	6-wk survival = 97% in e-TIPSS *vs* 67% in control (NNT = 3.3). 1-yr survival = 86% in e-TIPSS *vs* 61% in control ($P < 0.001$). 1-yr re-bleeding = 3% in e-TIPSS *vs* 50% in control ($P < 0.001$, NNT = 2.1).	mean Child score = 9.4. mean MELD score = 16.2. About 50% of study participants had Child C cirrhosis. Majority had ALD. NSBB (propranolol or nadolol) was administered with EBL in 25 patients.
Lv *et al*[44], 2019 (China)	Child B and C < 14 points, regardless of active bleeding.	(1) Primary: Transplant-free survival; and (2) Secondary: Failure to control bleeding or rebleeding, new or worsening ascites, overt HE, and other complications of portal hypertension and adverse events.	6-wk TFS = 99% in e-TIPSS *vs* 84% in SOC ($P = 0.02$). 1-yr TFS = 86% in e-TIPSS *vs* 73% in SOC ($P = 0.046$; NNT = 8). 1-yr re-bleeding/uncontrolled bleeding = 11% in e-TIPSS *vs* 34% in SOC ($P < 0.0001$).	mean Child Score = 8.0. mean MELD score = 13.8. More than 55% patients had Child-Pugh B without active bleeding. 75% of patients had Hepatitis B and had Child B cirrhosis. No significant difference in the incidence of HE was observed between two groups.
Dunne *et al* [46], 2020 (United Kingdom)	Child B and C, 8-13 points (regardless of active bleeding at the endoscopy).	(1) Primary: 1-yr survival; and (2) Secondary: Survival at 6 wk, early rebleeding (within 6 wk) and late rebleeding (between 6 wk and 1 yr), and the development of HE.	1-yr survival = 79.3% in e-TIPSS *vs* 75.9% in SOC ($P = 0.79$). e-TIPSS group showed a trend to reduced variceal re-bleeding ($P = 0.09$).	Median Child score = 9.8. Median MELD score = 17. More than 90% of participants had ALD. More than 55% had Child-C disease. 23/29 received TIPSS, 13 within 72 h. 18/29 (62%) in SOC group had carvedilol, 3 had cardio-selective beta- blocker and 2 had rescue-TIPSS for early re-bleeding. Incidence of HE was higher in e-TIPSS group ($P < 0.05$).
Observational studies				
Garcia-Pagán *et al*[49], 2013 (Europe)	Child-B with active bleeding or Child-C < 14 points.	(1) Composite primary: Failure to control acute bleeding or to prevent clinically significant variceal rebleeding; and (2) Secondary: mortality, development of other complications related to portal hypertension and the percentage of follow-up days spent in hospital.	1-yr survival = 86 % in e-TIPSS *vs* 70% in SOC ($P = 0.056$); e-TIPSS had lower incidence of failure to control bleeding or rebleeding than patients receiving SOC (3 *vs* 15, P <0.001).	mean Child score = 10. mean MELD score= 17. No significant difference in incidence of HE. Incidence of development of new or worsening ascites was low in e-TIPSS group ($P < 0.01$).
Rudler *et al* [52], 2014 (France)	Child-C 10–13 cirrhosis or Child-B with active bleeding	(1) Primary: prevention of rebleeding at 1 yr; and (2) Secondary: 3 and 6-mo survival, liver transplantation, control of bleeding, rate of rebleeding at 6 wk, between 6 wk and 1 yr, and the occurrence of adverse events (HE, acute cardiac failure, sepsis).	1-yr survival = 71% in e-TIPSS *vs* 74% in control ($P = 0.77$). 1-yr free of rebleeding = 97% in e-TIPSS *vs* 51% in control ($P < 0.001$).	mean Child score = 11.2. mean MELD score = 21.5. 77% had ALD and 77% had Child-C cirrhosis. Patients with previous history of variceal bleeding or with PVT were also included.
Thabut *et al* [50], 2017 (France)	Child-C (< 14) or Child-B with active bleeding	Survival at 5-d, 6-wk and 1-yr.	1-yr survival = 85% in e-TIPSS *vs* 59% in control ($P = 0.04$).	67% had ALD. 52% undergoing TIPSS had Child C cirrhosis. 35% were eligible for e-TIPSS. Severity of liver disease was the only parameter that influenced survival.
Hernández-Gea *et al*[51], 2018 (Europe and Canada)	Child-C score (< 14 points) or Child-B plus active bleeding	(1) Primary: Survival at 6 weeks and 1 year; and (2) Secondary: (a) The composite end-point of failure to control acute bleeding (up to day 5), early rebleeding (from day 5 to day 42), and late rebleeding (from day 42);	6-wk survival = 92% in p-TIPSS *vs* 77% in control. Overall, 1-yr survival = 78% in p-TIPPS *vs* 62% in control ($P = 0.014$). 1-yr survival in Child C patients = 78% in e-TIPSS *vs* 53% in control ($P = 0.002$). 1-yr survival in Child-B + AB = 77% in	Median MELD score= 15.5. Median Child Score= 10. More than 75% of patients had ALD. Development of de novo or worsening of previous ascites was significantly less in p-TIPSS group ($P < 0.001$). No

		(b) onset or worsening of ascites; and (c) development of HE.	p-TIPSS *vs* 75% in control (P = 0.935).	difference in incidence of HE was observed in two groups.
Lv *et al*[45], 2018 (China)	Any grade of cirrhosis (with Child score < 14) and AVB.	(1) Primary: All-cause mortality; and (2) Secondary: Failure to control acute bleeding or rebleeding, new or worsening ascites and development of overt HE.	Overall 6-wk mortality = 3.6% in e-TIPSS *vs* 10.6 % in SOC (P = 0.002). Overall 1-yr mortality = 14.1% in e-TIPSS *vs* 17.3% in SOC (P = 0.218). e-TIPSS group had significantly lower mortality in MELD ≥ 19 category.	Patients with Child A cirrhosis were also included. Only small number (< 20%) had Child C cirrhosis. Survival benefit was not seen in Child B patients without active bleeding. Incidence of HE was not significantly different between two groups.
Trebicka *et al* [22], 2020 (Multicentre)	Child-C, Child-B with active bleeding.	(1) Primary: All-cause mortality or liver transplantation at 6 wk and 1 yr; and (2) Secondary: Rebleeding.	6-wk mortality = 13.6 % in e-TIPSS *vs* 51% in SOC group of patients with ACLF (P = 0.002). 1-yr mortality = 22.7% in e-TIPSS *vs* 56.5% in SOC group with ACLF (P = 0.002).	Patients with ACLF had a higher rate of rebleeding compared to patients without ACLF (42-d: P < 0.001; 1-yr: 22.9% *vs* 17.7%, P = 0.024).

e-TIPSS: Early transjugular intrahepatic portosystemic stent-shunt; RCT: Randomised controlled trial; HVPG: Hepatic venous pressure gradient; HCC: Hepatocellular carcinoma; PHTN: Portal hypertension; PVT: Portal vein thrombosis; TFS: Transplant-free survival; HIV: Human immunodeficiency virus; ICU: Intensive care unit; NSBB: Non-selective beta-blockers; EBL: Endoscopic band ligation; IGV: Isolated gastric varices; MELD: Model for end-stage liver disease; ALD: Alcohol-related liver disease; NNT: Number needed to treat; HE: Hepatic encephalopathy; SOC: Standard of care; ACLF: Acute on chronic liver failure.

in Figure 1 and Figure 2.

In García-Pagán *et al*[39] landmark RCT published in 2010, patients with Child-Pugh C < 14 or Child-Pugh B with active bleeding at index endoscopy were considered high-risk patients. While there is clear justification of including patients with Child C disease in this category[11], the selection of Child B patients (with active bleeding on endoscopy) was not very clear. The composite primary end point in their study was of failure to control acute bleeding or to prevent clinically significant variceal rebleeding within 1 year. Their trial of 63 patients showed that early covered TIPSS (placed within 72 h of index bleeding) not only reduced re-bleeding at 1 year (3% *vs* 50%, P < 0.001) but also improved 6-wk [97% *vs* 67%; absolute risk reduction = 30%; number needed to treat (NNT) = 3.3]; and 1-year survival rates (86 *vs* 61%, P < 0.001; NNT = 4.0) in high-risk patients with cirrhosis when compared to standard of care (NSBB plus EBL). It is important to note that rates of treatment failure and death were higher in Child C patients than in those with Child-B disease, and mortality rates in Child B category did not appear to be significantly different statistically between SOC and TIPSS arms (2/16 *vs* 1/16). However, the trial was not powered enough to conduct appropriate subgroup analyses[39]. Patients with prognostic factors unlikely to benefit from TIPSS placement were excluded from this trial and the subsequent studies (Table 1).

In the light of emerging evidence, subsequent guidelines incorporated the use of pre-emptive or e- TIPSS, as a treatment option in patients with AVB at high risk of treatment failure[40-43] (Table 2).

An RCT from China included 132 cirrhotic patients who were randomly assigned (2:1) to receive pre-emptive TIPSS or standard of care (NSBB + EBL)[44]. This RCT showed better 1-year transplantation-free survival (primary outcome) in e-TIPSS group than in the control group; with greatest benefit for those with a MELD score between 12 and 19 (P = 0.04, NNT = 8)[44]. However, all patients with Child B and C (< 14 points) cirrhosis were included regardless of active bleeding at the index endoscopy. Secondly, the patient demographics were significantly different from other studies and most patients were Child-Pugh B without active bleeding (57%). Over 75% of patients had Hepatitis B related cirrhosis. Only 43% of patients were high risk according to the previously described criteria[39], and considered to benefit from e-TIPSS intervention[45]. Therefore, absolute risk difference of 13% for 1-year (transplant-free) survival in e-TIPSS group appeared to be lower than in the previous RCTs (34% and 25%)[39]. There was no significant difference in incidence of hepatic encephalopathy between the two groups.

A recently published RCT from the UK included 58 patients with a Child-Pugh score of 8-13, without previous treatment for portal hypertension related bleeding, regardless of active bleeding on endoscopy[46]. Patients were randomised to receive e-TIPSS or standard of care (carvedilol + EBL). There was no difference in 1-year survival rate (primary outcome) between the SOC and e-TIPSS groups (75.9% *vs* 79.3% respectively, P = 0.79). More than 90% of participants had alcohol related liver disease and majority (over 80%) were actively consuming alcohol at inclusion reflecting real Western world population. Over 55% had Child-C disease with median MELD score of 17, comparable with Garcia-Pagan study[39]. In the e-TIPSS group, 23/29 patients

Table 2 Summary of current Guidelines regarding early transjugular intrahepatic portosystemic stent-shunt

Ref.	Guidelines	e-TIPSS recommendations
[40]	Baveno VI Consensus Workshop (2015)	An early TIPSS (p-TIPSS) with PTFE-covered stents within 72 h (ideally < 24 h) must be considered in patients bleeding from EV, GOV1 and GOV2 at high risk of treatment failure [*e.g.*, Child-Pugh class C < 14 points or Child-Pugh class B with active bleeding) after initial pharmacological and endoscopic therapy (1b; A)]. Criteria for high-risk patients should be refined.
[41]	American Association for the Study of Liver Diseases (2017)	In patients at high risk of failure or rebleeding (CTP class C cirrhosis or CTP class B with active bleeding on endoscopy) who have no contraindications for TIPSS, an "early" (pre-emptive) TIPSS within 72 h from EGD/EVL may benefit selected patients.
[42]	The European Association for the Study of the Liver (2018)	Early pre-emptive covered TIPSS (placed within 24–72 h) can be suggested in selected high-risk patients, such as those with Child class C with score < 14 (I; 2). However, the criteria for high-risk patients, particularly Child B with active bleeding, remains debatable and needs further study. Up to 10%–15% of patients have persistent bleeding or early rebleeding despite treatment with vasoactive drugs plus variceal ligation, and prophylactic antibiotics. TIPSS should be used as the rescue therapy of choice in such cases (I; 1).
[43]	British Society of Gastroenterology (2020)	In patients who have Child's C disease (C10-13) or MELD ≥ 19, and bleeding from oesophageal varices or GOV1 and GOV2 gastric varices and are hemodynamically stable, early or pre-emptive TIPSS can be considered within 72 h of a variceal bleed where local resources allow (weak recommendation, moderate quality of evidence). However, large multi-centre randomised controlled trials are necessary to determine whether patients with Child's B disease and active bleeding or with MELD 12-18 benefit from early pre-emptive TIPSS.

PTFE: Polytetrafluoroethylene; p-TIPSS: Pre-emptive transjugular intrahepatic portosystemic shunt; EV: Oesophageal varices, GOV: Gastro-oesophageal varices; CTP: Child-Turcotte-Pugh; EGD: Oesophago-gastro-duodenoscopy; EVL: Endoscopic variceal ligation; MELD: Model for end-stage liver disease.

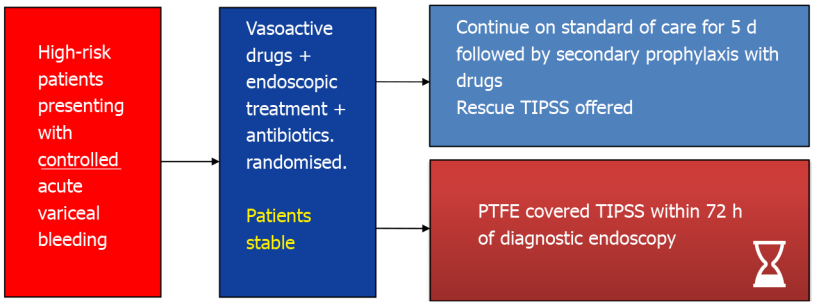

Figure 1 Early transjugular intrahepatic portosystemic stent-shunt – study design. High risk criteria: Child's C or Child's B + active bleeding, Child-Pugh score 8-13, Child's B + C; Maximum threshold: CPS > 13; TIPSS: Transjugular intrahepatic portosystemic stent-shunt; PTFE: Polytetrafluoroethylene.

(79%) actually underwent TIPSS and only 13 within 72 h, but all within 5 days. There was no difference in worsening or new ascites, with more encephalopathy (46.1% *vs* 20.7%, $P < 0.05$) and a trend towards lower variceal rebleeding in the e-TIPSS group ($P = 0.09$). Notably, previous RCT[39] and recent individual data metanalysis[47] did not show significant difference in development of hepatic encephalopathy between the two groups. Though the study was not powered enough to reach valid conclusions, it demonstrated better survival in the SOC arm than the previous European RCT[39] (76% *vs* 61%), although SOC survival rate is comparable to the Chinese RCT[44].

Better survival in SOC group could be explained by improved initial management of AVB (vasoactive drugs, antibiotics and endoscopic band ligation), with better access to intensive care. Furthermore, carvedilol was used to a greater extent. The improved SOC could be major factor in the lack of difference in survival between the two groups. In the SOC arm, 18/29 patients received carvedilol (at a median dose of 6.25 mg a day). Carvedilol with its additional alpha-1 antagonism profile, seems to have greater effect on reducing HVPG than other traditional NSBB (propranolol and nadolol) and may have a beneficial effect in SOC group but this needs further validation. This study has led to much debate in relation to patient selection[48].

Observational studies

The benefits of e-TIPSS have been shown by several (but not all) observational studies. Most of these studies used similar clinical high-risk and exclusion criteria as the study by Garcia-Pagan[49] (Table 1). It is important to note that in a French national audit and in large multicentre study, only a minority of patients eligible for e-TIPSS (according to defined criteria) actually received e-TIPSS (6.7% and 9.8% respectively) [50,51]. Survival benefit of e-TIPSS was only seen in those with Childs-Pugh C disease

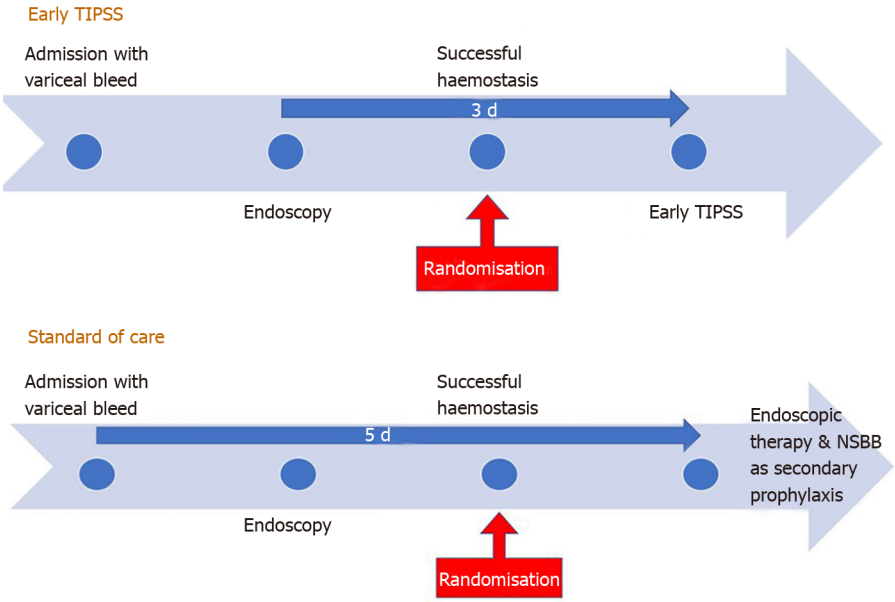

Figure 2 Design of early transjugular intrahepatic portosystemic stent-shunt and standard of care. TIPSS: Transjugular intrahepatic portosystemic stent-shunt.

in a large multicentre study including 671 patients[51]. These large observational multicentre studies underscore the lack of adherence of physicians to concept of e-TIPSS and difficulty in arranging e-TIPSS (within limited timeframe) in a real-life practice. Most physicians did not believe in the role of e-TIPSS. Two European studies did not find a statistically significant increase in survival in e-TIPSS group[49,52]. One of these studies included patients with Child-Pugh score up to 15 points i-e, patients with significantly advanced liver disease[52].

A recent observational study from China included 1425 patients with cirrhosis and variceal bleed[45]. Most of the patients had cirrhosis due to viral hepatitis and e-TIPSS was also offered to Child-Pugh A patients and Child-Pugh B patients without active bleeding. Survival benefit was observed in patients fulling the high-risk criteria used in Garcia Pagan RCT[39] and with MELD score ≥ 19 but not in patients with Child-Pugh A or Child-Pugh B without active bleeding.

In a recently published retrospective study, e-TIPSS has also shown improved 6-weeks and 1-year survival ($P < 0.05$) in patients with ACLF[22]. 671 patients were eligible for e-TIPSS and only 66 received e-TIPSS. 22 out of 66 e-TIPSS patients had ACLF. However, the findings need to be interpreted with caution due to the small sample size and require validation in larger prospective studies.

Systemic reviews and meta-analyses

A few metanalyses of studies looking at the role of early TIPSS in patients with AVB have been published in recent years. A well-designed meta-analysis of two earlier RCTs[39] and two observational studies[49,52] comparing e-TIPSS with standard of care showed that e-TIPSS is associated with reduced overall mortality (odds ratio = 0.38, 95%CI: 0.17-0.83, $P = 0.02$)[53] (Table 3). It is important to note that sensitivity analysis looking separately at Child B patients with active bleeding and those with Child C (< 14 score) showed that survival benefit was only observed in Child C (< 14 score) patients but not so in Child B patients. There was also significant reduction in rebleeding with e-TIPSS without significant difference in incidence of hepatic encephalopathy. Moderate heterogenicity was observed among the studies and the recent RCTs by Lv *et al*[44] and Dunne *et al*[46] were not included in this metanalysis. The authors concluded that further study was required to identify factors associated with poor outcome after e-TIPSS.

A recently published individual patient data meta-analysis assessed the efficacy of e-TIPSS in high-risk patients[47]. They included 7 studies: 3 randomized controlled trials[12,39,44] and 4 observational studies[45,49,51,52] comprising 1327 patients. As discussed previously, one of the RCTs[44] and one of the observational studies[45] included patients in all Child-Pugh categories, therefore only individual data of those patients fulfilling the current high-risk criteria (Child-Pugh B with active bleeding and Child- Pugh C up to 13 points) were included in this individual meta-analysis. This

Table 3 Early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: Key meta-analyses

Ref.	Design	Results	Comments
Deltenre *et al*[43]	4 studies (2 RCTs[12,39] and 2 Observational[49,52]) included.	e-TIPSS was associated with fewer deaths [odds ratio (OR) = 0.38, P = 0.02], and with lower rates of bleeding (OR = 0.08, 95%CI: 0.04–0.17, P < 0.001) within 1 year when compared to SOC, without increase in incidence of encephalopathy (OR = 0.84, 95% CI: 0.50–1.42, P = 0.5).	There was moderate heterogeneity between studies. No significant difference in mortality was observed between Child–Pugh B and C patients. This could be explained by inclusion of sicker patients (C-P score < 14) in Rudler *et al* [52] study.
Nicoară-Farcău *et al*[47]	Individual data meta-analysis from 7 studies (3 RCTs[12,39,44] and 4 observational studies[45, 49,51,52]), comprising 1327 patients (310 received e-TIPSS, 1017 received SOC (drugs + endoscopic treatment).	Overall, e- TIPSS significantly increased 1- year survival compared with SOC [hazard ratio (HR) 0.443; P < 0.001]. e-TIPSS significantly reduced the risk of failure to control bleeding/preventing variceal rebleeding (HR = 0.338; P < 0.001) and ascites without increasing risk of HE, compared with SOC.	Only individual data of those patients fulfilling the high-risk criteria (Child-Pugh B with active bleeding and Child-Pugh C < 14 points) from included studies were included. On multivariate analysis patients with Child-Pugh score > 7 points had a significantly worse survival than those with Child-Pugh score ≤ 7. Both prospective and observational studies were included and latest UK RCT[46] and the multicentre French audit[50] were not included.
Tripathi *et al*[54]	3 RCTs[39,44,46] comparing e-TIPSS (with covered stent) with SOC, comprising 152 patients.	e-TIPSS significantly reduced incidence of re-bleeding (RR = 0.20; P ≤ 0.001). Improvement in overall survival at 1 and 2 yr was not statistically significant between two groups (RR = 0.62; P = 0.16 and RR = 0.62; P = 0.19 respectively).	There was no significant difference in incidence of HE. RCTs are underpowered to reach firm conclusion about the survival benefit of e-TIPSS.

e-TIPSS: Early transjugular intrahepatic portosystemic stent-shunt; RCT: Randomised controlled trial; SOC: Standard of care; HE: Hepatic encephalopathy.

meta-analysis showed overall survival benefit of e-TIPSS over standard of care. Six-week and 1-year survival were significantly higher in the e-TIPSS group than in the SOC group (93% *vs* 76.8% and 79% *vs* 62%, respectively P < 0.001). Moreover, benefit of e-TIPSS was observed in both CP- B patients with active bleeding (P = 0.008;) and in CP-C patients (P < 0.001). Number of patients needed to treat to save one life was 4.23 (95%CI: 3.57–6.94). Multivariate analysis showed that patients with a CP score > 7 points had a significantly worse survival than those with CP score of 7 points or less. e-TIPSS significantly reduced the risk of failure to control bleeding/preventing variceal rebleeding (P < 0.001) in all patients. Moreover, risk of developing new or worsening ascites was significantly reduced by the e-TIPSS in the overall population (P < 0.001). This meta-analysis showed no significant differences in the risk of developing hepatic encephalopathy in the overall population (P = 0.553). However, a limitation of this meta-analysis is the inclusion of both prospective and observational studies, and the authors concluded that further prospective studies are necessary. The latest UK RCT[46] and the multicentre French audit[50] were not included in this meta-analysis, thus somewhat limiting its utility.

A recent meta-analysis of 152 patients in three prospective RCTs, including the latest UK RCT[39,44,46], concluded that e-TIPSS is more effective in preventing variceal rebleeding than standard of care (EBL and medical management) without increase in adverse events[54]. e-TIPSS with covered stents significantly reduced incidence of bleeding (RR = 0.20, 95%CI: 0.09–0.42, P < 0.001). This was associated an improvement in overall survival, but it did not quite reach statistical significance, at 1 and 2 years (RR = 0.62, 95%CI: 0.33–1.19 and RR = 0.62, P = 0.16 95%CI: 0.31–1.26, respectively). Incidence of hepatic encephalopathy was similar across the studies[54].

FUTURE DIRECTIONS

Patients with advanced liver disease i-e with Child-Pugh C score (up to 13 points) and MELD score ≥ 19 benefit from the e-TIPSS intervention in the described studies. However, benefit of this intervention in patients with less severe disease i-e with Child-Pugh B or MELD < 19 is not very robust and there is further need to define the high-risk category.

Though patients with child score > 13 points are considered too sick for early TIPSS intervention with high mortality, it is not very clear from the literature if there is a maximal threshold of severity of liver disease beyond which there is no benefit from e-TIPSS intervention. Indeed certain patients with ACLF may benefit from e-TIPSS following AVB. This concept needs further revalidation in a multi-centre trial

collecting large numbers of patients.

Outcomes after an episode of variceal bleed have improved in the last decade with improved 1-year survival in patients receiving standard care[44,46] as compared to the earlier landmark RCT[39], causing reluctancy to adopt e-TIPSS approach among the practicing physicians. Moreover, providing e-TIPSS (within 72 h of admission) is challenging in most healthcare systems, even in centres providing 24/7 TIPSS service, and is a significant barrier to adoption of e-TIPSS. Indeed, recruitment in trials[39,46] was very slow and careful reading of the manuscripts suggests that the included patients may not be truly representative of the entire population of patients with severe cirrhosis and variceal bleeding. With such stringent inclusion criteria, the applicability of this therapeutic approach is questionable in a larger cohort of cirrhotic patients.

Even if TIPSS was performed outside the 72 h window, so called "late e-TIPSS", it may not have a significant impact on the outcomes given the time frame for acute bleeding is 5 d as defined by the Baveno consensus[40]. Indeed, benefits of e-TIPSS placement following oesophageal variceal bleeding have been observed for up to 28 d after index endoscopy[55,56]. Therefore, a more pragmatic approach to the time window for e-TIPSS is an important consideration when designing future trials.

CONCLUSION

The role of e-TIPSS in acute variceal bleeding requires further prospective study with adequately powered trials. Studies should focus on careful patient selection, investigate optimal timing of TIPSS, and explore quality of life and health economics.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7625-7648

DOI: 10.3748/wjg.v27.i44.7625

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

REVIEW

COVID-19: Effect on gastroenterology and hepatology service provision and training: Lessons learnt and planning for the future

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Author contributions: Anjum MR led with literature search and submission of the article; Anjum MR and Chalmers J drafted the article; Chalmers J, Hamid R, and Rajoriya N contributed to editing of the article; Hamid R and Rajoriya N did critical appraisal of the article; Rajoriya N is the guarantor of the article.

Conflict-of-interest statement: All authors have no conflict of interests to declare.

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

In late 2019, reports arose of a new respiratory disease in China, identified as a novel coronavirus, severe acute respiratory syndrome coronavirus 2. The World Health Organisation named the disease caused by the virus 'coronavirus disease 2019 (COVID-19)'. It was declared a pandemic in early 2020, after the disease rapidly spread across the world. COVID-19 has not only resulted in substantial morbidity and mortality but also significantly impacted healthcare service provision and training across all medical specialties with gastroenterology and Hepatology services being no exception. Internationally, most, if not all 'non-urgent' services have been placed on hold during surges of infections. As a result there have been delayed diagnoses, procedures, and surgeries which will undoubtedly result in increased morbidity and mortality. Outpatient services have been converted to remote consultations where possible in many countries. Trainees have been redeployed to help care for COVID-19 patients in other settings, resulting in disruption to their training - particularly endoscopy and outpatient clinics. This has led to significant anxiety amongst trainees, and risks prolongation of training. It is of the utmost importance to develop strategies that continue to support COVID-19-related service provision, whilst also supporting existing and future gastroenterology and Hepatology services and training.

Received: March 27, 2021
Peer-review started: March 27, 2021
First decision: June 14, 2021
Revised: June 28, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 28, 2021

P-Reviewer: Zelai NT
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

Changes to healthcare provision during the pandemic have generated new and improved frameworks of service and training delivery, which can be adopted in the post-COVID-19 world, leading to enhanced patient care.

Key Words: COVID-19; Gastroenterology; Hepatology; Training; Service provision

Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has led to adverse effects on many aspects of life. Healthcare professionals have faced unique challenges with COVID-19 including surges in cases with easing of lockdown measures, the emergence of new variants, and the roll-out of mass vaccination. The pandemic has had a largely negative impact on gastroenterology and hepatology service provision and training across the world. These difficulties have affected job-roles and training across the medical profession. We review the available evidence on the COVID-19 disruption to gastroenterology and hepatology service provision and training, discussing recommendations to minimise the interference going forward.

Citation: Anjum MR, Chalmers J, Hamid R, Rajoriya N. COVID-19: Effect on gastroenterology and hepatology service provision and training: Lessons learnt and planning for the future. *World J Gastroenterol* 2021; 27(44): 7625-7648
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7625.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7625

INTRODUCTION

In December 2019, China's regional World Health Organisation (WHO) office was informed of a new respiratory disease of unknown cause[1], detected in Wuhan, Hubei Province, China. It was characterised as a novel coronavirus strain, severe acute respiratory syndrome coronavirus 2; the WHO subsequently designated the disease caused by the virus as 'coronavirus disease 2019 (COVID-19)'. The virus spread rapidly and a Public Health Emergency of International Concern was declared at the end of January 2020, followed by pronouncement of a global pandemic in March 2020. The WHO asked for global preparedness to detect and manage COVID-19[1], urging countries to ensure they had adequate hospital resources and functional test-and-trace systems. The epicentre of the pandemic shifted to Europe in March 2020 and cases began to rise exponentially in the United Kingdom. The first United Kingdom case of COVID-19 was in a 75-year-old lady on February 21, 2020, identified through a retrospective analysis of sputum samples by the University of Nottingham[2]. At the time of writing, COVID-19 has claimed over 128000 Lives in the United Kingdom[3] and over 3.9 million worldwide[4]. These figures from United Kingdom pertain to individuals that died within 28 d of a positive test; they do not consider the likely substantial number of indirect deaths. With the global spread of COVID-19, there has been an unprecedented impact on healthcare services. Wards and intensive care units (ICUs) have been inundated with COVID-19 patients, and healthcare staff have been redeployed from their base specialities to help care for COVID-19 patients. Resource scarcity, in addition to the risk of virus transmission with face-to-face interaction means that speciality services have been decimated. In the field of gastroenterology and hepatology, many different aspects of service provision have been affected such as a significant reduction in diagnostic and therapeutic endoscopy. Out-patient clinics have been cancelled or moved to remote consultations; liver transplantation programmes have been temporarily halted or limited to only super-urgent transplantation at times. These changes, combined with the redeployment of trainees to COVID-19 wards and ICUs, have resulted in restricted gastroenterology and hepatology training, and an associated impact on trainees' morale. This article will review the available evidence regarding gastroenterology and hepatology service provision and training during the pandemic. We will provide an overview of the disruption caused by the COVID-19, and also review recommendations to minimise the impact of this disruption. The United Kingdom will be used as a reference, as it is

familiar to the authors; other countries will be compared and contrasted throughout. At time of writing, the United Kingdom healthcare system is dealing with a further peak of infections (January/February 2021).

THE EFFECT OF COVID-19 ON GASTROENTEROLOGY AND HEPATOLOGY SERVICES

An overview of the disruption

Worldwide, most countries imposed national lockdowns to tackle rising COVID-19 cases. In the United Kingdom, this was first implemented on March 23, 2020. In the NHS most elective medical and surgical work was halted in an attempt to increase availability of bed spaces for COVID-19 patients requiring hospital admission. Staff were redeployed to departments with increasing pressures - primarily ICUs, Acute Medicine and Respiratory wards with doctors often required to fill other staffing vacancies such as ICU nursing. In gastroenterology and hepatology, the effects from COVID-19 have not been discriminatory; all ranks of staff, from junior doctors to consultants, have been affected[5-8]. In the United Kingdom, the British Society of Gastroenterology (BSG) issued consensus guidelines on endoscopy service provision, and the management of specific disease patient cohorts, including: Inflammatory bowel disease (IBD), chronic liver disease, immunosuppressed patients, coeliac disease, and on transjugular intrahepatic portosystemic shunt service provision[9]. Similarly, gastroenterology and hepatology associations and experts worldwide have issued consensus opinions and guidelines regarding the management of patients during the pandemic[10-15]; which will be expanded upon throughout this article.

Endoscopy

During the peak of the pandemic, a number of major gastroenterology societies worldwide advised to postpone non-urgent examinations[16] including: The European Society of Gastrointestinal Endoscopy[17], the World Endoscopy Organisation[18], the American College of Gastroenterology, the American Society for Gastrointestinal Endoscopy[19], the Canadian Association of Gastroenterology[20], joint statements from Indian Gastroenterology societies[21], and the Gastroenterological Society of Australia[22]. Recommendations from major organisations have been summarised in Table 1.

In conjunction with other position statements and expert opinions[16,17,23,24], the BSG issued a statement during the first wave of the pandemic stating that 'all non emergency Gastrointestinal (GI) endoscopic procedures should stop immediately'[25], for an initial period of six weeks. The recommendation included the Bowel Cancer Screening Programme (BCSP), symptomatic two-week-wait (2WW) and Urgent Suspected Cancer (USC) referrals. The statement concluded that very few patients were likely to come to harm from the pause, highlighting that the lack of ICU beds and potential risk of higher surgical mortality during the initial COVID-19 peak may in fact cause harm if endoscopy was continued as before. The BSG accepted that a small number of patients may have a delay in their diagnosis[26]; and advised that 2WW and USC referrals should be individually triaged and risk-assessed by gastroenterology consultants. An 'Urgent Deferred Waiting List' was created, so as to prioritise follow-up and investigations when services resumed. Of note, urgent 2WW referrals by primary care physicians, primarily for patients with suspected cancer, decreased by up to 80% during the first peak of the infections[27]. Endoscopy service disruption occurred worldwide. Alboraie *et al*[7] obtained data from 163 participating centres, across 48 countries and 6 continents. The majority (93.9%) of the centres were hospital-based endoscopy units, affiliated with teaching hospitals. The centres reported a significant reduction in their procedural numbers during the pandemic: 85% reported that procedure volume decreased by > 50%, with only emergency services being continued, and four endoscopy units (2.45%) completely suspended procedures. The top three indications for endoscopic procedures included upper GI bleeding (89.6%), lower GI bleeding (65.6%), and cholangitis (62.6%)[7]. A study from Melbourne, Australia also demonstrated that significantly fewer procedures were performed during the peak of COVID-19, as compared to the pre-pandemic era[28]. A survey of 123 North American gastroenterology practices, comprising of 1379 Gastroenterologists in 32 United States states and 4 Canadian provinces observed a 90% decrease in endoscopy volume during the COVID-19 pandemic[29]. A further survey of 252 centres from 55 countries, reported a consistent reduction in endoscopic activity across all continents[30]. The United Kingdom's BSG issued guidance on the resumption of

Table 1 Summary of advice on Endoscopy service provision during the peak of COVID-19 pandemic from major societies around the world (March-April, 2020)

World Endoscopy Organization[18]
Postpone routine and elective procedures.
Take patient temperature at presentation, and screen for travel to high risk area, contact with COVID patient and occupational exposure.
Upper GI procedures (OGD, EUS, ERCP) carry highest risk of aerosolization.
Colonoscopy and flexible sigmoidoscopy carries some risk of aerosols generation.
In a positive patient or those at high risk of COVID, only perform highly urgent/emergent procedures.
Use enhanced PPE during Upper GI procedures, and standard PPE with surgical mask during lower GI procedure but use enhanced PPE if available or if high risk patient.
Perform GI endoscopy in negative pressure room if available.
If, endo-tracheal intubation required, perform in negative pressure room and minimize staff in the room during intubation.
Standard endoscope reprocessing is sufficient to kill COVID virus.
Essential person only in the room to conserve PPE.
Consider pre-procedure COVID testing for risk stratification.
European Society of Gastrointestinal Endoscopy[17]
Postpone all elective and surveillance endoscopy.
Case by case triage for non-urgent/emergent procedures.
Appropriate training of staff on the infection prevention strategies for COVID.
Health Care Professionals in endoscopy units should be triaged daily for sign symptoms of COVID and tested if needed.
COVID can effectively be inactivated by commonly used disinfectants having virucidal activity, so, reprocessing of flexible endoscopes and endoscopic accessories should be performed according to published guidelines.
Cleaning the endoscopy unit with virucidal agents is recommended as infection by contact is possible.
If feasible, online care should be provided (e.g. telemedicine) for pre-procedure clinics and assessment.
Before procedure, both patient and health care professional to use surgical face mask and face shield/visor if available. Temperature check all patients.
Relatives and caregivers should not have access to the GI endoscopy unit.
For patients who are considered at high risk for COVID, separate pre- and post-GI endoscopy recovery areas(or timeslots) should be arranged.
Same enhanced personal protection measures are recommended for all procedures, both upper or lower GI endoscopies for simplification.
Use negative pressure procedure rooms if available for high risk or positive COVID patients.
Post-procedure, consider tracing and contacting patients at 7 d and 14 d to inquire about any new COVID diagnosis, or development of COVID symptom.
American Society for Gastrointestinal Endoscopy[19]
Postpone on urgent procedures.
On arrival patients have their temperature checked and screened for COVID symptoms, contact or travel history.
Guidance on use of PPE.
Use negative pressure rooms if available.
Reprocessing of endoscopes as per standard guidelines.
Contact patient 14 d after the procedure to inquire about any COVID symptoms.
British Society of Gastroenterology[25,31]
All non-emergency GI endoscopic procedures should stop immediately, including Bowel Cancer Screening and fast-track referrals.
All emergency upper GI endoscopic procedures are classified as AGPs, irrespective of the COVID status of the patient, because the virus can be shed before any symptoms are present.
All staff in the room should wear PPE.
Case by case triage of cancer suspicious and other referrals.
Maintain a separate Urgent Deferred Waiting List to prioritise their proactive follow-up and investigation when services resume.

Subsequent guidance recommended to consider pre-procedural symptom screen and COVID testing with separation of high risk COVID sites from COVID minimised sites for low risk patients.
Indian Society of Gastroenterology[21]
Postponed routine non-urgent procedures
Screen patients pre-procedures with symptoms screen, travel and contact history.
Take temperature of all patients pre-procedure.
Minimum number of staff in the procedure room.
Use appropriate PPE based on risk assessment and stratification.
Standard disinfection processes are effective against COVID.
Surgical masks for patients' use too, if they have respiratory symptoms.

AGP: Aerosol-generating procedure; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; GI: Gastro-intestinal; OGD: Oesophago-Gastro-Duodenoscopy; PPE: personal protective equipment.

GI endoscopic services in the United Kingdom[31] during the recovery phase after the pandemic's first peak in 2020. In addition to restoring specialist staff, it was advised that there needed to be increased infection control to minimise peri-procedural infection spread[32], including additional time and space for procedures, secure supplies of personal protective equipment (PPE), and the need for COVID-19-minimised facilities, where COVID-19 positive patients, are separated from those that are unlikely to have the infection. To accurately separate patients, individuals were screened for symptoms and tested for COVID-19 prior to endoscopy, with COVID-19 patients often deferred to the end of an endoscopy list, or indeed their procedure undertaken in a different hospital area. Similar guidelines were issued by gastroenterology organisations in other parts of the world, *e.g.*, America[33], Europe[17] and Asia[34]. A multi-centre prospective study of COVID-19 transmission in 6208 patients having outpatient endoscopy, found low prevalence of transmission peri-endoscopy if performed in a 'COVID-19-minimised pathway', which consisted of symptom screening and/or a COVID-19 reverse transcriptase polymerase chain reaction (RT-PCR) swab of patients prior to procedure. In total, 2611 patients in the study had a COVID-19 swab pre-procedure, and only 3 tested positive, all of whom were asymptomatic. None of the patients developed symptoms of COVID-19 within two weeks after the procedure based on telephone follow-up[35]. It should be noted that data for this study was collected during the recovery phase of the first peak in United Kingdom (Summer 2020), when community prevalence of COVID-19 was low. In contrast, Alboraie *et al*[7] found that 25.8% of participating centres (from 48 countries) reported positive cases of COVID-19 amongst patients within two weeks of their procedure date, although the percentage of positive cases within each individual centre was not reported. The data, however, included patients requiring urgent endoscopy and was not limited to COVID-19-minimised sites, which may have accounted for the higher infection rates. GI endoscopic procedures generate significant aerosol[36]. Therefore, the importance of air flow was imperative, as it was recognised that post-procedure turnover time or 'down-time' was required to let aerosolised particles settle and therefore reduce potential cross-infection. The down-time is dependent on air flow cycles within the procedure room to reduce the particulate burden of air, the procedure type and the Covid status of patient[37]. Infection control reviews of some units revealed suboptimal air exchange in endoscopy rooms and a lack of infection prevention training in some units. A survey of 83 institutes by Hungarian Society of Gastroenterology revealed 33.33% participants had infection prevention training, less than 1% said that they have negative pressure procedure rooms, and only 20% reported that they have some form of mechanical ventilation or air purification system[38]. It was not easy to implement all the recommendations quickly with resultant delays in resumption of endoscopic services. These operational issues and others, such as: redeployment of endoscopy staff, reconfiguration of endoscopy units/ service pathways, pre-procedure triage of patients and COVID-19 testing, and enhanced PPE significantly added to the workload of staff.

Colorectal cancer services

The COVID-19 pandemic has had a major disruptive impact on colorectal cancer service provision. Deviation from the national guidelines was observed at every point

in the patient care pathway including referrals, endoscopy/colonoscopy, surgery provision, surveillance and patient follow-up[39]. The degree of disruption varied between units both in the United Kingdom[40] and internationally[39,41]. Following cessation of non-emergency endoscopy and BCSP during the first wave of COVID-19, Rutter *et al*[42] analysed the United Kingdom's 'National Endoscopy Database'[42], and demonstrated that endoscopic activity reduced significantly compared to pre-COVID-19 Levels by an average of 12%, but declining to 5% at the lowest point (Figure 1). The authors reported an increase in the per-procedure cancer detection rate, owing to labour-intensive triage of the existing endoscopy waiting lists and of new referrals by senior clinicians. Despite this, the weekly number of cancers detected decreased by an average of 58% compared to pre-COVID, suggesting 72% of colorectal, 37% of oesophageal, 52% of gastric, and 19% of pancreato-biliary cancers may have been missed. This was a substantial and concerning reduction in cancer detection due to decreased endoscopic activity. Reduced procedure rates were confirmed by other studies, with reports of decreased adherence to colorectal screening programmes[39], and an 81% drop in colonoscopic activity, during the pandemic[7]. Maringe *et al*[43], modelled the impact of diagnostic delays on patient survival in the United Kingdom, and estimated that there would be 15.3%-16.6% additional deaths in colorectal cancer patients, and an increase of 5.8%-6.0% in deaths by oesophageal cancers. This data highlighted the severe consequences of reduced endoscopy services during the COVID- 19 pandemic, with substantial foreseeable increases in the number of avoidable cancer deaths, as a result of these diagnostic delays. A cross-sectional study from the United States assessed the weekly number of newly identified breast, colorectal, lung, pancreatic, gastric and oesophageal cancers during the COVID-19 pandemic. The authors found that detection fell 46.4% (from 4310 to 2310) for the 6 cancers combined[44].

Management of liver diseases and liver transplantation

COVID-19 affected liver transplant services throughout the world. In the United Kingdom, 7 adult and 3 paediatric centres performed 8740 Liver transplants performed in the United Kingdom in the last ten-year period[45]. National Health Service Blood and Transplant and the United Kingdom Liver Advisory Group updated and reviewed guidance throughout the pandemic. Liver transplantation centres were advised to continue procedures on a case-by-case basis - largely for patients in a clinically urgent category [based on high 'United Kingdom Model for End-Stage Liver Disease' scores or hepatocellular carcinoma (HCC) on verge of going out of transplant criteria]. Where possible, activity was continued for all super-urgent liver transplantation. Age for donation after brainstem death was restricted to 60 years and donation after circulatory death restricted to 50 years on March 23, 2020, with a projected transplant reduction of 51%[46]. After further appraisals, these donor criteria were relaxed later in the 2020. During the peaks of COVID-19, some centres temporarily suspended all liver transplant activity (except for extremely super-urgent cases) due to a surges in hospital admissions, shortages of ICU beds, and organ procurement restrictions implemented by the organ donation authority[47,48]. The number of liver transplantations in the United Kingdom fell by 84% (3 nationally per week) during the first COVID-19 peak[46]. On average, the number of patients on the United Kingdom liver transplant waiting list at the end of each month between January 2019 to March 2020, was above 400. This number fell rapidly to 109 during the first peak, by May 2020, with only clinically urgent patients being left on the waiting list and the remaining patients temporarily suspended[46]. At the time of writing, the United Kingdom again has severe restrictions in place for liver transplantation activity, due to a further surge of COVID-19 infections (February 2021). Analyses from the National Organ Procurement Agency in France, and the United Network for Organ Sharing in the United States, in April 2020, demonstrated a reduction in solid-organ donor transplantations by 90.6% and 51.1% respectively[49]; this was predominantly due to fewer kidney transplants, but there was also a substantial decrease in liver, lung and heart transplants. The American Association for the Study of Liver Diseases (AASLD) recommended postponing liver transplantation, advising each programme to consider its capability regarding ICU beds, ventilator availability and blood donation[50]. The Saudi Association for the Study of Liver Diseases and Transplantation published a position statement, advising that efforts should be made to persevere with normal transplant activity, but with the adoption and flexibility of individual transplant pathways, including virtual tele-medicine consultation to avoid patient contact and stricter preventive measures[51]. The European Association for the Study of the Liver (EASL) advised that centres should prioritise patients who have a poor short-term prognosis without liver transplant, including those with acute liver

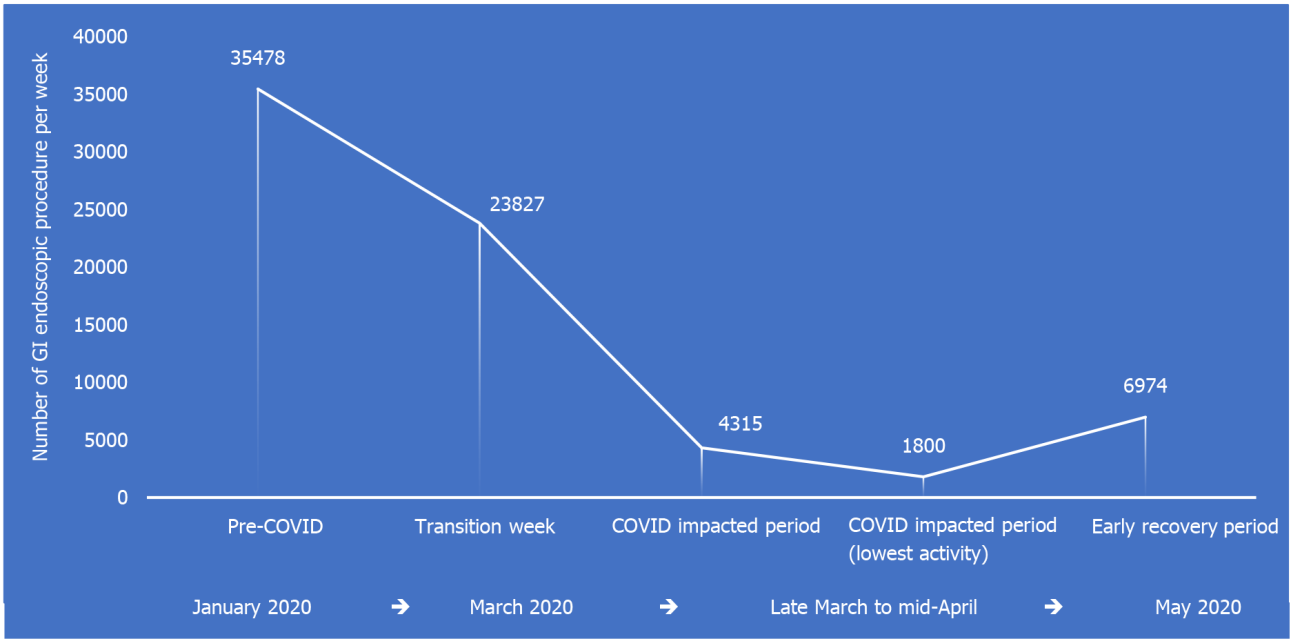

Figure 1 Reduction in United Kingdom wide endoscopic activity (cumulative number of procedures/week) during 1st peak of COVID-19 infections. Source: United Kingdom's National Endoscopy Database analysis, Rutter *et al*[42].

failure, high MELD score and HCC[52]. The liver transplant society of India recommended a moratorium on all non-urgent transplants during the initial COVID-19 peak[53]; similarly, the Pan-Arab Association of Gastroenterology recommended that non-urgent transplants should be postponed[54]. Hepatology societies across the world provided guidance for the management of patients with liver diseases in both inpatient and outpatient settings, and on liver transplant (Figure 2). The AASLD[55], EASL[56] and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)[13] all advised: To separate COVID-19 positive and negative in-patient cohorts with liver disease, minimise contacts by reducing staff levels during ward rounds, limit the number of investigations performed and have restrictions on visitors. Similarly, for outpatients, the societies recommended that units offer remote consultation for appointments and only perform essential investigations. Bollipo *et al*[10] summarised these recommendations including advice on the management of liver transplant, endoscopy, liver cancer, and both inpatient and outpatient care. A multicentre, retrospective, cross-sectional study by Amaddeo *et al*[57] found that fewer patients with HCC presented to the multidisciplinary meetings, and had a treatment delay that was longer in the COVID-19 period than in 2019.

IBD services

Outpatient IBD services were severely impacted during the pandemic with cancellations of clinic appointments and subsequent conversion to remote consultations[58]. IBD surveillance endoscopy services were temporarily halted as a non-urgent endoscopic procedure. This has affected the follow-up of known IBD patients, as well as new IBD cases. With regards to new patients, cases were triaged to assess urgency and consideration made to delay diagnostic endoscopy and imaging in those with mild symptoms and moderate biomarkers[59]. It was recommended that initiation of therapy be on a case-by-case basis. Known IBD patients were advised to continue their current medications given the increased risk of infection associated with active disease and hospitalisation. Similar guidelines were issued by other IBD societies around the world. The European Crohn's and Colitis Organisation advised that centres adopt virtual consultations, use a home faecal calprotectin test for monitoring, and limit endoscopic evaluations to those patients in whom it was felt to be absolutely necessary [60]. Recommendations to minimise contact and reduce endoscopic intervention were echoed in guidance from the International Organization for the study of Inflammatory Bowel Disease and Crohn's and Colitis Foundation of America[61]. Healthcare providers adopted and modified their IBD unit protocols to minimise both patient-patient and patient-health care worker contact. Centres also screened patients for COVID-19 symptoms before they attended infusion units[62]. A significant proportion of new IBD patients were diagnosed and treated without having endoscopic or

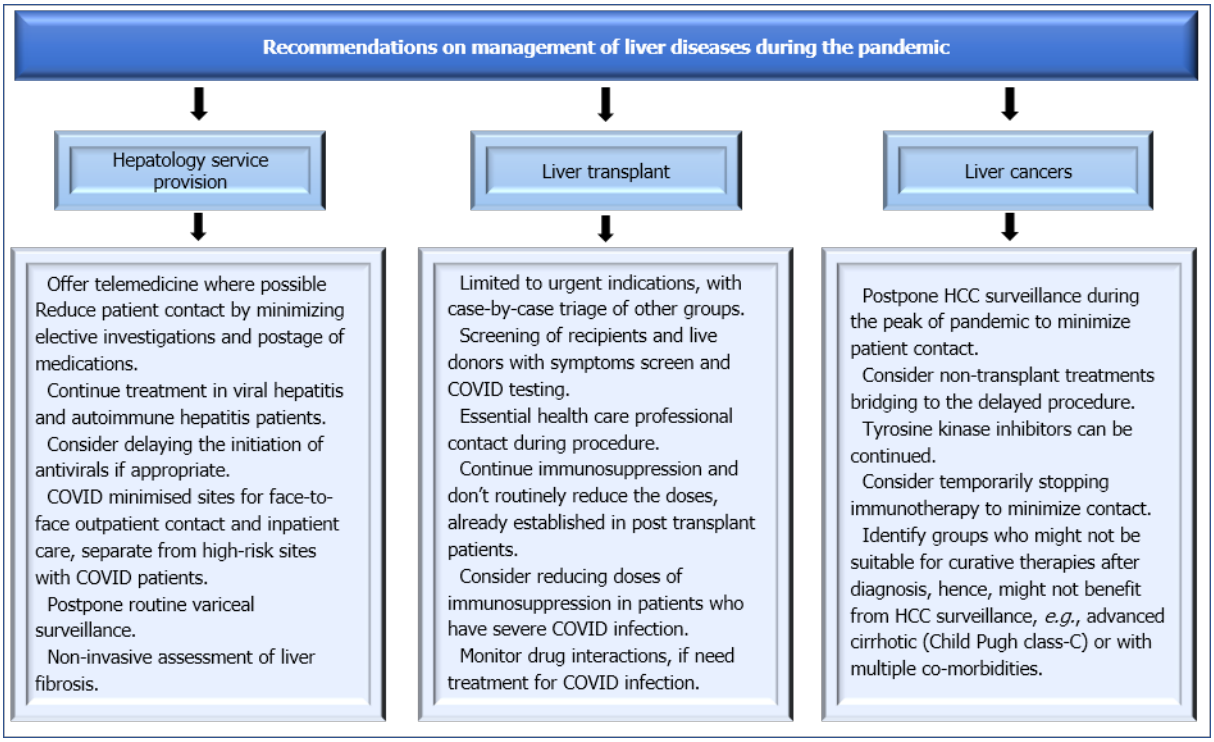

Figure 2 Summary of recommendations from major Hepatology societies across the world on management of liver diseases during the pandemic. Sources: American Association for the Study of Liver Diseases[55]; British Society of Gastroenterology[9]; European Association for the Study of the Liver[56]; Indian Gastroenterology Society[21]; International Liver Transplantation Society[10]; Pan-Arab Association of Gastroenterology[54]. HCC: Hepatocellular carcinoma.

histological evaluation[63]. Based on early observations from China and Italy, IBD patients were felt to be at higher risk of severe COVID-19, particularly older patients with comorbidities, patients on high doses of systemic corticosteroids, and patients with active disease[64,65]. The United Kingdom's BSG issued guidance to stratify IBD patients into low, medium and high-risk of a poor outcome from COVID-19 infection [59]. High-risk patients included those on: (1) Concomitant therapies (immunomodulator and biologic) who were over 70 and/or had selected co-morbidities; (2) Those who were receiving daily steroids (≥ 20 mg prednisolone or equivalent); (3) Those who had recently commenced biologics with immunomodulators or systemic steroids; and (4) Those who had short bowel syndrome and required nutritional support or were on parenteral nutrition. IBD patients identified as high-risk were advised to shield by the United Kingdom government during the peaks of the pandemic; they were advised to stay home wherever possible and only to leave the house for essential reasons including medical appointments. This risk assessment was consistent with findings from Brenner *et al*[66] of 525 international COVID-19 cases reported to the 'Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease' (SECURE-IBD) registry[66]; 50% of IBD patients with a severe COVID-19 outcome (*i.e.*, ICU admission, ventilator use, and/or death) were over 70 years of age, and 50% of those who died had cardiovascular co-morbidities. The SECURE-IBD registry was also analysed to assess COVID-19 outcomes with various IBD medications. The major finding from these studies[66,67], was that thiopurine treatment, as both monotherapy and combination therapy, was associated with an increased risk of severe COVID-19 infection when compared with tumour necrosis factor alpha (TNF-α) antagonist monotherapy. Further findings were that TNF-α antagonist monotherapy may have a protective effect against severe COVID-19 and that no significant differences were observed in outcomes when comparing classes of biologics[67]. This data was consistent with previous findings that thiopurines increase the risk of viral infections compared with TNF-α antagonists[68]. This was reflected in real-world practice; Sharma and Meade[58] assessed prescribing of IBD medications in a United Kingdom tertiary hospital and found increased de-novo biologic therapy compare to pre-pandemic, particularly among thiopurine-naïve patients. The BSG recommended: not to stop current medications (to avoid any flares of IBD), to consider anti-TNF monotherapy if needed and to avoid immunomodulators[59]. A panel of 15 IBD experts convened to review the management of acute severe ulcerative colitis

(UC) in the context of COVID-19, recommending that patients with UC flares should be isolated throughout their hospital stay, and Infliximab along with steroids should be considered as a rescue therapy if needed. It was not deemed appropriate to commence thiopurine therapy for maintenance however steroids and Infliximab continuation was considered more appropriate[69]. Provision for follow-up and investigations to monitor disease response in IBD patients was impacted throughout the pandemic, providing a further rationale for initiating treatment with more efficacious agents earlier. Kennedy *et al*[70] surveyed 125 IBD services during the first peak of COVID-19 infections and found that there was a significant reduction from baseline in whole-time equivalent gastroenterologists and IBD nurses providing elective outpatient care. Of concern, 27% of services reported no access to faecal calprotectin, and a further 32% reported reduced access to faecal calprotectin testing [70], making management more challenging. Curtailment in IBD-specific services, *e.g.*, outpatient services, endoscopic services, regular multidisciplinary meetings to discuss complex patients and re-deployment of specialist nurses was accompanied by an increase in IBD helpline queries from patients[70]; 94% of services reported an increase in IBD helpline activity. In summary, worldwide, organisations made significant changes to ensure safe care provision to the IBD population. These adaptations included: diagnosing IBD clinically in several instances without endoscopic, histological and in most cases radiological investigations, remote prescribing/ Laboratory investigations and medicine infusion arrangements outside of high risk COVID-19 main hospital sites along with provisions of tele-consultations for patients [63,70,71]. Due to rapid changes in IBD patient services, governance structures for development of novel ways of working remains a major focus. Changes to IBD delivery structure should be discussed with patients where possible. In a survey of 685 IBD patients[72], participants reported that the COVID-19 pandemic has had a negative impact upon their psychological well-being and quality of life. The patients reported an increase in perceived stress with 39% of respondents worried about their IBD care, but respondents were happy with delivery of care remotely.

Outpatient gastroenterology and hepatology clinic provision

Outpatient clinics were disrupted by COVID-19, with temporary suspension of many routine outpatient clinics, and with conversion of standard outpatient face-to-face clinic consultations to telephone or virtual appointments - referred to as "telemedicine". Emergency clinic appointments were provided depending on clinical urgency, with some centres adopting temporary email addresses or telephone helplines for patients to contact the hospital. As the first wave subsided, face-to-face appointments were re-introduced in some centres, depending on local COVID-19 policies; however, telemedicine has now been adopted and sustained by many gastroenterology and hepatology outpatient services including viral hepatology and IBD clinics. The advent of telemedicine preceded the pandemic[73], but its use has increased exponentially in the past year, with a study from New York[74] reporting an 8729% increase in the use of video consultations during COVID-19, accounting for 21.9% of outpatient visits. Worldwide, medical organisations issued guidance regarding the use of telemedicine[75-77] outlining recommendations for establishing telemedicine systems, as well as ethical considerations. Broadly, telemedicine is considered appropriate for patients with straightforward complaints, who do not require physical examination, feel comfortable using the required technology, and who can be provided with all necessary information and prescriptions remotely. Key principles regarding patient identification, capacity, consent and safe information storage hold true for telemedicine as they do for in-person consultations. A multi-modal telemedicine network was established in Sichuan Province, Western China, in January 2020, in response to the COVID-19 pandemic[78]. The network synergised a new 5G service, a smartphone application, and an existing telemedicine system; it was funded in the short-term by disaster funds. An expert group was established to provide education to medical staff. By March 23, 2020, the authors reported that 9085 patients had received online consultations or interventions through the application, and 1094 *via* telephone. Four hundred and twenty-four consultations were conducted for severe and critical COVID-19 patients highlighting the substantial potential of telemedicine. In addition, radiologists used the network to successfully perform remote computed tomography (CT) scanning of 152 patients, allowing quality imaging in areas with a severe shortage of qualified technicians. Various other organization have issued guidelines for remote consultations[79-81]. Key principles include: Correct identification of patient at start of teleconsultation and use of available medical records/referral letters in conjunction with patient history to decide whether a safe management plan can be made remotely, or is face to face consultations required. Any

remote examinations requires patient consent, with the United Kingdom's GMC recommending the use of a chaperone during video consultation in a same way as one would do during face-to-face consultation[82]. Medicolegal organizations have also generated their recommendations on tele-consultations on similar basic principles[83]. Similar recommendations to the United Kingdom GMC guidelines were issued for physicians by Ghosh *et al*[84] in India. The American Telemedicine Association has described similar principals and framework of remote consultations even before the pandemic[85]. The impact of telemedicine has been studied by Lee *et al*[86] in the setting of liver transplantation in a randomised prospective trial; 106 patients were randomised to standard of care face-to-face practice, or telemedicine home-based care - utilising tablets, video-calls, and texts. Participation rates, quality of life and 90-d hospital re-admission rates were compared, with lower re-admission rates at 90 d (28% *vs* 58%, $P = 0.004$) and improved quality of life with regards to physical function and general health in the telemedicine group. Munroe *et al*[87] designed a single-arm, crossover study during the COVID-19 pandemic. A telemedicine consult, *via* phone or video call, was offered to patients referred to the practice; patients could accept a virtual consult or request a review in person. The authors reported high levels of patient acceptance of telemedicine, and no discernible changes in outcomes or care-use related to medical decision-making, time to appointment or patient satisfaction. In a retrospective observational study from New York, United States, Ramaswamy *et al*[74] analysed feedback from telemedicine consultations pre- and mid-pandemic; in both cohorts, satisfaction was significantly higher with video consultations vs. in-person visits (94.9% *vs* 92.5%, $P < 0.001$). McKenna *et al*[88] surveyed 212 general neurological patients from Dublin, Ireland who had attended remote consultations and found that 76% of patients felt remote consultations were either "just as good" (67.1%) or "better" (9.0%) than face-to-face consultations. Those who reported remote consultations to be 'not as good' were older (52.3 years *vs* 46.6 years, $P = 0.045$) and had neurological conditions that required clinical examination (66.7%) or an undiagnosed condition awaiting investigation/ review (46.7%). The United Kingdom's Medical Protection Society assessed doctors' opinions on telemedicine[89]; of 1250 respondents 70% agreed that the benefits of telemedicine were 'unquestionable' during the pandemic. However, 80% feared that the doctor-patient relationship could 'break down' or were worried telemedicine might deny some patients' treatment. Almost three-quarters of doctors expressed concern about medically missing something in a remote consultation and 60% were worried about a claim or investigation.

THE EFFECT OF COVID-19 ON GASTROENTEROLOGY AND HEPATOLOGY TRAINING

Worldwide, COVID-19 has had a disruptive effect on the training of gastroenterology and hepatology doctors due to redeployment and cancellation of educational activities. This has been compounded in some settings by medical staff exhaustion and burnout. Most routine and non-emergency specialty services have been suspended, including a drastic reduction in endoscopic activity, and hence training opportunities for gastroenterology and hepatology trainees. Global shortages of PPE led to 'non-essential persons' being excluded from endoscopic lists[90] to conserve the supply in some hospitals. In addition, to minimise risk of infection, centres were advised to consider restricting staffing for procedures, limiting endoscopy to a small number of specialist consultants, and excluding trainees[91]. A survey of Australian gastroenterology trainees demonstrated a 75% reduction in endoscopic activity, with 30% of trainees prohibited from performing emergency endoscopy; again, to limit staff exposure and to conserve PPE[92]. Most training assessments, specialty exams, and continuous professional development (CPD) activities were cancelled during the first peak of COVID-19. In May 2020, the BSG surveyed its members and found that 66% of United Kingdom trainees were not doing any specialty clinics, 29% were unable to continue their formal research commitments, and 53% were unlikely to achieve their Annual Review of Competency Progression targets, which would therefore risk prolongation of training[93]. In a survey of Canadian gastroenterology trainees[94], 94% were concerned about achieving and/or maintaining clinical competence, and 71% were concerned about prolongation of training due to the pandemic. During the pandemic, trainees have experienced an increase in workload, often in new clinical environments, leading to exhaustion and burnout. This, alongside missed training opportunities, has generated anxiety and stress amongst specialty trainees. In an international survey of 770 endoscopy trainees from 63 countries[95], 52.4% of respondents reported anxiety

and 18.8% reported burnout. Cravero *et al*[96] surveyed 1420 Internal Medicine trainees from the United States, China, Saudi Arabia, Taiwan and other countries[96] ,and found that the trainees caring for COVID-19 patients were more likely to have worked additional hours compared to pre-pandemic, and that the incidence of reported burnout was proportionate to the number of COVID-19 patients that trainees had cared for. Fifty-nine percent trainees expressed concerns about their preparedness for independent practice while 20% trainees reported that pandemic has negatively effected the progress towards their career goals. Karampekos *et al*[97] surveyed Greek gastroenterology fellows, and fellowship programme directors regarding the impact of COVID-19 on endoscopy training. The two groups broadly agreed on the factors associated with a negative impact: an unknown timeframe of COVID-19 measures, cancellation of endoscopy, and fewer endoscopies performed by fellows. The fellows were significantly more concerned about their ability to acquire and/or maintain endoscopy competence than their programme directors (83.1% *vs* 27.8%, $P < 0.001$). In addition, proposed strategies to address training post-pandemic varied with fellows predominantly suggesting prolongation of training (49.4%) and programme directors suggesting an increase in daily workload (44.4%). This study highlighted the importance of involving both trainees and programme leaders in planning and decision-making for training during and after the COVID-19 pandemic. Finally, remote working (*e.g.*, telemedicine) has allowed some flexibility in doctors' working patterns - clinics can be performed from offices or from home with remote access to patient data. This was actively encouraged by some hospitals to reduce clinician footfall within the hospital during the COVID-19 pandemic. However, there was a resultant effect on training with fewer opportunities for direct learning and an inability to gain immediate, face-to-face advice from supervisors.

PROPOSALS FOR GASTROENTEROLOGY AND HEPATOLOGY SERVICE-PLANNING AND TRAINING PROVISION DURING THE COVID-19 PANDEMIC AND BEYOND

There has been a significant increase in the caseload of COVID-19 patients in recent months, and it is clear that the pandemic is far from over. In addition to direct COVID-19-related morbidity and mortality, there has been significant collateral damage, due to disruption in routine and urgent health services, such as cancer workload. After the 1st wave, the rate of COVID-19 related admissions slowed and the services began to recover. However with mutation of the virus, surges in COVID-19 cases recurred with most countries thereafter experiencing a 2nd and some 3rd waves. At time of writing, the delta-variant is on the increase in the United Kingdom. With experience gained in planning for surge capacity in hospitals, reconfiguration of services has been easier for many in the 2nd wave and now 3rd wave. Services however are constantly running at high volume, often exacerbated by winter pressures (in Europe) meaning healthcare services remain at constant risk of being overwhelmed. In this section, we discuss recommendations to ensure optimal continuity of patient care and gastroenterology/hepatology training during the pandemic (Figure 3), and indeed new approaches could continue well after the pandemic has ended. It should be reinforced that general principles of adequate PPE, social-distancing measures, robust contact-tracing systems and the roll-out of COVID-19 vaccines remain crucial pillars for controlling COVID-19 on mass population levels.

Protecting the workforce

Adequate PPE and infection-control measures are essential to allow staff members to remain safe whilst caring for COVID-19 patients. A study of 420 healthcare workers deployed to care for COVID-19 patients in Wuhan, China[98], demonstrated that effective infection prevention measures protected the workforce from getting infected - despite working an average of 100-130 h in the ICU, none of the participants reported symptoms of COVID-19 and all remained antibody negative. The study highlighted the importance of the procurement and distribution of PPE, as well as providing adequate training to healthcare professionals in its use. Thomas *et al*[99] appraised global PPE guidance and the available scientific evidence regarding aerosols, virus transmission, and respiratory protection. The authors concluded that there were shortcomings with the Public Health England's PPE guidelines and recommended urgent revision to protect the United Kingdom's NHS workforce during the pandemic. Rising infections and deaths amongst healthcare workers worldwide prompted calls

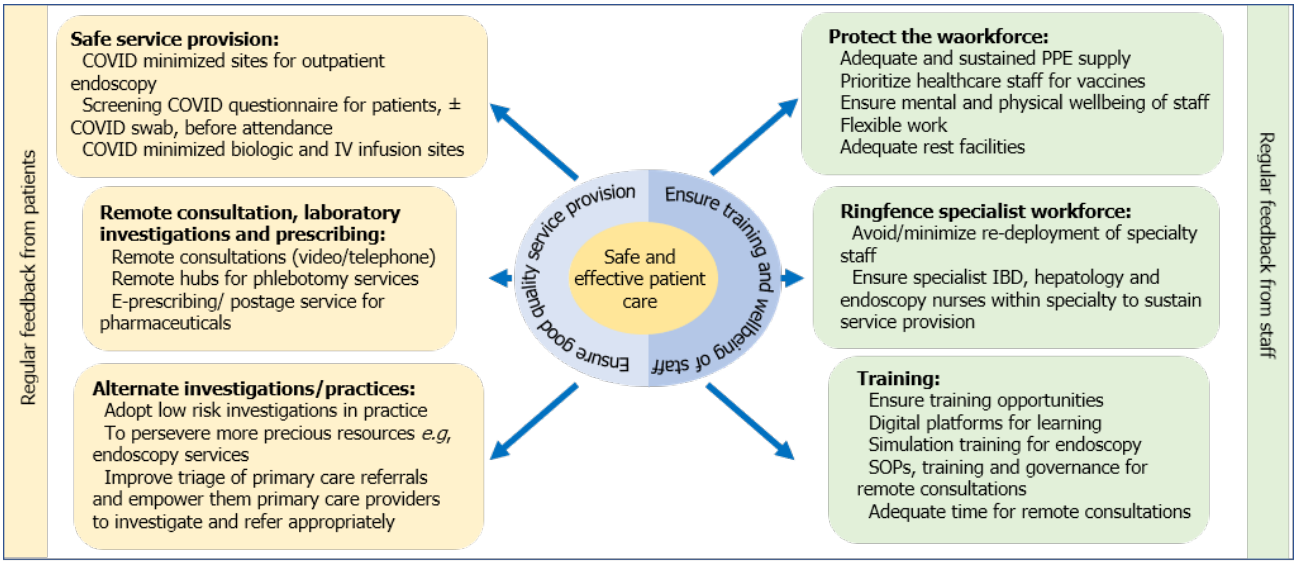

Figure 3 Recommendations for safe and effective care provision and continuation of training during Pandemic. IBD: Inflammatory bowel disease; PPE: Personal protective equipment; SOP: Standard operating procedure.

for urgent action and PPE provision[100-103]. In parallel to PPE provisions, the vaccine rollout remains key in protecting the workforce. In many countries, healthcare staff have been identified at a high-risk group and targeted for vaccination early. This is in conjunction with medically at-risk patients and also the elderly. Mental and physical wellbeing of healthcare workers is required for optimal performance, and health care organisations and medical unions should have resources in place. Hospital organisations should ensure where possible, that staff have adequate breaks on shifts, and there are adequate provision of rest facilities, especially given 'social-distancing' requirements. It is increasingly recognised that the pandemic will have a psychological impact on the majority of healthcare staff, and support must therefore be put in place [104]. Early experiences from China[105], and more recently from Europe[106], suggest that healthcare staff are likely to experience negative mental health outcomes due to the pandemic. In addition, with the increased workload burnout is becoming more common[96]. In the United Kingdom, NHS England recommended support and flexibility for staff working during the pandemic[107]. Tomlin *et al*[106] proposed a phased model of the mental health burden, in which stressors from different phases of the pandemic are considered and coping strategies were suggested for both the individual and the organisation for each stage. For example, in the preparation phase individuals should be aware of anxiety levels and stress triggers, and the organisation is advised to identify those who may experience challenges to their mental wellbeing. This may be particularly relevant to those with existing mental health difficulties, those with caring responsibilities and those who have recently survived a stressful or traumatic experience. By identifying vulnerable staff members and putting resilience and well-being plans in place, hospital organisations can take a proactive approach to supporting staff thereby minimising harm.

Ring-fence the specialist workforce

During surges of COVID-19 infections, much of the specialist workforce was redeployed to care for COVID-19 inpatients. Initially, it was assumed that a short period of disruption in services would cause minimal harm; however, more recent data has highlighted significant missed and delayed diagnoses as a result[42,43]. In subsequent waves of infection, a proportion of the specialist workforce where possible should aim to continue working within the specialty; for gastroenterology and hepatology this would include doctors, specialist nurses and endoscopy staff, all of whom are essential to sustain the components of the service - in-patient caseload, out-patient clinics and endoscopy lists. A balance between ongoing service provision for the COVID-19 pandemic and that of existing and ongoing services for gastroenterology and hepatology must be found.

COVID-19-minimised sites

The objective of 'COVID-19-minimised' sites, also known as 'cold' sites, is to

physically segregate COVID-19 patients from those that are not infected, ideally on separate sites (cold *vs* hot sites). This allows units to provide endoscopic and outpatient services to gastroenterology and hepatology patients while minimising their risk of COVID-19 contacts. The United Kingdom's BSG proposed a telephone screening questionnaire, termed the 'SCOTS criteria'[108]; 3-7 d prior to endoscopy, the patient is asked if they have symptoms of COVID-19 or have come into close contact with a known or suspected case in the preceding 14 d. They interviewer should also consider supplementary factors such as the patient's occupational risk of exposure, recent travel from a known risk area, and if that patient is in a shielded category. For patients reporting any of the SCOTS criteria, clinicians should consider if the procedure can be delayed for 14 d or if an alternative can be offered. If not, level 2 PPE should be used and procedure performed in a 'hot' location. Screening should be combined with a COVID-19 test pre-procedure. Kim *et al*[109] performed a meta-analysis of 19 studies, predominantly from China, to assess the diagnostic performance of RT-PCR, the commonly used COVID-19 screening test; they reported a pooled sensitivity of 89%. The negative predictive value reduced as COVID-19 prevalence increased, ranging from 99.9% with 1% prevalence to 93.4% at 39% prevalence. Despite this variation, the data suggests that combining a screening questionnaire with an RT-PCR swab prior to procedure would allow accurate triage of patients to either COVID-19-minimised or high-risk sites for endoscopy. COVID- 19-minimised sites ensure safe service provision and enhance patient confidence in attending healthcare facilities during the pandemic. With the advent of vaccinations, COVID-19 passports for visits for endoscopic procedures or to hospital may be considered when risk stratifying patients, however remains to be clarified based on long-term immunity data and risk of infection thereafter.

Training and governance for telemedicine

Despite advances in technology, telemedicine has been a novel concept for a substantial proportion of health care workers. There was no formal training or governance structure in place in many hospitals due to the speed of rollout of this modality during the pandemic. It is recommended, especially for junior trainees, that remote consultations are undertaken at a location where a consultant is available for opinion and supervision (*i.e.*, hospital clinic rather than from home). This would provide the structured approach and will enable trainees and supervisors to have close liaison and immediate contact for questions. Another format proposed could be mixture of face-to-face and remote consultations within a same session. This would ensure social distancing for visiting patients at intervals while having teleconsultations in between in appropriate patients. There are pros and cons to remote clinics[110]. The pros include working remotely both for clinician and patient without the need for travel thus minimizing COVID-19 contact risk and preserving PPE used during face to face consultations. Remote clinics also acts as initial triage of patients to identify those who would benefit from face-to-face consultation. Cons include no clinical examination and a lack of visual clues. Issues also may exist contacting patients or conversing with those not speaking native language to the healthcare professional. It is important to consider patients who either can't use or don't have access to the internet or telephone resources. Consultation from all stake holders is required before starting a new remote consultation service, otherwise conflicts may arise. One recent example was that Government of India launched the 'e-sanjeevani', a national teleconsultation service during the pandemic while Indian Medical Association issued an advisory against the use of telemedicine in most situations, creating confusion amongst medical community[111]. Appropriate allocation of time and planning is required for both to face-to-face consultations and remote consultations in terms of time allocations for appointment and job planning[112]. Clinical exam might not be performed for remote consultations but other logistic issues like arranging investigation requests and prescriptions are important part of remote consultations and require allotted time. From the patient perspective, these remote consultations should be booked and organized as timed slots as one would expect with face-to-face consultations, rather than them being called at random time or date.

Exploring alternative investigations and practices

Using evidence-based practices, alternative investigations and modified pathways (Figure 4) may have a role to replace some of the more resource-intensive services and tests.

Primary care referral triage: Advanced triage of primary care referrals is not a new concept. An example of this was The Royal Wolverhampton Trust, United Kingdom,

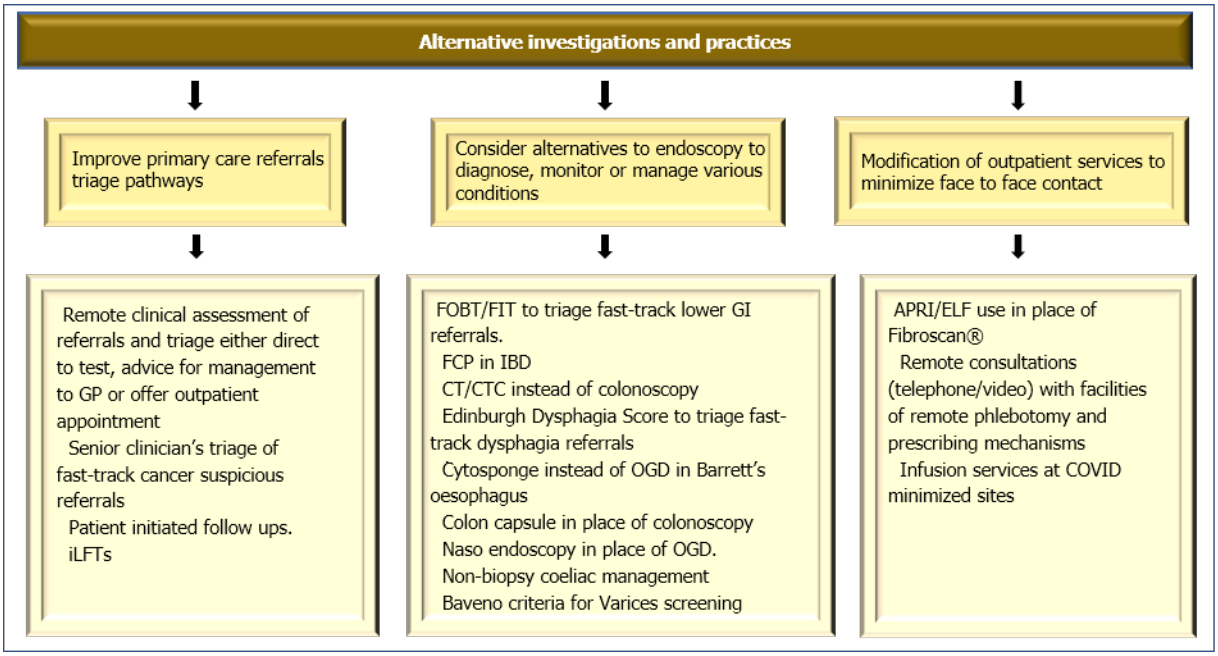

Figure 4 Recommendations for exploring alternative investigations and practices during the pandemic. FOBT: Faecal occult blood test; FIT: Faecal Immunochemical Test; FCP: Faecal Calprotectin; CTC: CT colonography; iLFT: intelligent liver function testing;APRI: aspartate aminotransferase to platelet ratio index; ELF: Enhanced Liver Fibrosis; OGD: Oesophagogastroduodenoscopy.

developing a 'Clinical Assessment Service' (CAS) in 2014-2016[113]. A Gastroenterologist reviewed primary care referrals, arranged investigations if necessary, and either discharged the patient back to primary care with advice, or arranged an outpatient appointment. The authors reported that 32% of triaged CAS patients were managed without the need for an outpatient appointment. In the first three years of using CAS, 3136 fewer outpatient appointments were required, which translated into a 481613 GBP cost saving. A Californian study of Rheumatology referrals reported similar results with 1/4 of e-referrals being resolved without a clinic appointment over a 4-year period[114]. The COVID-19 pandemic has demonstrated a need for widespread development of such services. During the pandemic, triaging has been used for 2WW and USC referrals; there is scope for this to be expanded to include many other primary care gastroenterology and hepatology referrals. Another concept is 'patient-initiated' follow-up or review, which has been pioneered by NHS Scotland (United Kingdom) during the pandemic[115]. For certain patients - those who are stable or maintained on long-term treatment, an alternative approach can be offered, in which the patient requests reviews based on their wants and needs, rather than being allocated routine appointments. Caveats exist for this method: patients require clear guidelines for when to request reviews and they must be able to confidently self-manage their condition. As a result a patient-initiated approach is unlikely to be suitable for certain cohorts *e.g.* alcoholic liver disease patients. In March 2019, Whear *et al*[116] conducted a meta-analysis of 17 randomised trials assessing patient-initiated follow up in patients with chronic health conditions. The authors demonstrated that patient-initiated appointment approach had little or no effect on patient anxiety/depression and patient satisfaction scores, when compared with consultant-led appointment systems. The need to adopt such frameworks in routine practice has become crucial during the pandemic, to minimise contact and to prioritise service provision for those who need it most urgently.

Endoscopy: Endoscopy services have been disrupted and limited during the COVID-19 pandemic, and since services have resumed, there is added cost to each procedure due to associated infection-control procedures and PPE. Therefore, alternative pathways and resources have been trialled, to triage patients and streamline services. Faecal occult blood or Faecal immunochemical tests (FITs) have been used to triage 2WW lower GI suspected cancer referrals[117]. In 2019, six United Kingdom 'FIT pioneer sites' shared data regarding FIT positive and negative cancers, in a combined 9182 patients[118]. The number of FIT negative cancers was 0.01%-0.75%, with a negative predictive value of at least 99.05%. The incidence of FIT-negative cancers was

highest in those with iron deficiency anaemia, suggesting that alternative methods of triage may be required in these patients. A pre-pandemic single centre study from Scotland, United Kingdom of 5422 patients[119], assessed the use of FITs in significant bowel disease (SBD) including colorectal cancer, high risk adenoma and IBD. The findings suggested that use of FIT in conjunction with a full blood count and clinical assessment correctly identified 93.9% of all SBD. There were fewer referrals to secondary care (15.1% reduction) and an increased yield of SBD detection (13.9% to 20.5%), suggesting that patients were appropriately targeted for colonoscopy. Of those not immediately referred, only 0.7% were found to subsequently have SBD. Widespread implementation of FIT testing may therefore provide an effective and safe way to identify patients at risk of SBD. Faecal calprotectin is an effective tool to distinguish between IBD and functional GI disorders[120]. It also correlates well with endoscopic and histological disease activity in known IBD patients[121], and therefore is a suggestion that serial faecal calprotectin measurement could be used to monitor disease activity and to detect relapse early. During the pandemic, CT-colonography (CTC) could be used preferentially to optical colonoscopy to screen patients for colorectal cancer[122]. CTC imaging is acquired by a single healthcare worker, and requires only a limited duration of close proximity with the patient. It is performed with disposable equipment and there is minimal exposure to stool. Such radiological alternatives may therefore reduce the risks associated with COVID-19 and preserve PPE. Staffing requirements would be minimised, but it should be noted that radiology services may also see increased caseloads with surges in COVID-19 infections. Validated clinical questionnaires could replace previous initial evaluation pathways and 'direct to test' endoscopy referrals during and following the pandemic. The Edinburgh Dysphagia Score[123] uses six parameters to stratify patients into high and low risk of cancer: age, sex, weight loss, duration of symptoms, location of dysphagia and acid reflux. In 435 patients, it was found to have a sensitivity of 97.5% in correctly stratifying cancer patients to the high-risk group; 30% of referrals were stratified to the low risk group and could therefore be investigated less urgently. The Eckardt Score could be used for evaluation in achalasia[124]. Cytosponge is a nonendoscopic, ingestible, sampling device and may provide a non-AGP alternative for diagnosis of Barrett's oesophagus[125] during the pandemic. It has also been shown to have potential as a triage tool for endoscopy, in patients with mild to moderate dysphagia with suspected oesophageal cancer[126]. Similarly, colon capsule endoscopy is an ingestible device that allows visualisation of the bowel without attendance at hospital. It is equally effective when compared to colonoscopy for identifying polyps more than 10mm in size and more sensitive than radiological investigations in the detection of colorectal cancers[127]. It may be an effective and safe alternative to colonoscopy during the pandemic[128]. New methods to triage and stratify patients who require endoscopic procedures could significantly reduce the demands on this service. However, the clinical effectiveness of using these new approaches will need to undergo rigorous testing and trials to ensure patients with significant pathology are not experiencing delays to endoscopy, or indeed being missed altogether. The United Kingdom's BSG updated guidelines on polyp surveillance towards end of 2019[129] that could mean reduction in number of surveillance colonoscopy procedures[130]. The units needed to validate their existing waiting lists in view of this updated guidance but pandemic hit in early 2020. It is imperative that validation work continues, especially when there is additional backlog of procedures when the activity was reduced during the pandemic. Non-biopsy protocol for coeliac disease diagnosis could also avoid need for endoscopy in patients who are symptomatic and IgA TTG ≥ 10 × upper normal limit on two occasions or one positive IgA TTG accompanied by positive endomysial antibodies (especially in children). Clinicians should be mindful of proceeding with requesting endoscopy though, if there are alarm symptoms present or if the patients belong to older age groups (≥ 55 years)[131]. It is important to incorporate alternative pathways as much as possible, as a modelling study from United Kingdom suggests that even with mitigation measures, it may take till after 2022 to clear backlog of endoscopic procedures[132].

Hepatology: Routine (non-urgent) Fibroscan® services were deferred in many hospitals during the peak of the COVID-19 pandemic[133]. Tests such as the Enhanced Liver Fibrosis test[134] or AST to Platelet Ratio Index[135] could be used as an alternative to assess fibrosis remotely. For patients with cirrhosis for whom endoscopy was recommended prior to the COVID-19 pandemic, for screening and surveillance of varices, it was suggested to initiate non-selective beta blocker (NSBB) therapy based on clinical judgement, taking into account Child Pugh class and platelet count[136].

Similarly, in patients with advanced fibrosis or cirrhosis, or at high risk of having portal hypertension and varices, the BSG advised to consider starting NSBBs treatment prophylactically during the pandemic[136,137]. The Baveno criteria[138] could be used to identify patients at low risk of having varices, though this would require Fibroscan services to be operational; arguably easier and associated with a reduced risk of COVID-19 transmission *vs* traditional endoscopic services. AASLD advised to postpone HCC surveillance from 6 mo to 8 mo in most cirrhotic patients who do not have key risk factors for HCC development[139]. The BSG[140] and EASL[13] also advocated delaying HCC surveillance during the peak of infections. Mehta *et al*[141] reviewed guidelines from various hepatology societies and provided recommendations on HCC surveillance and monitoring. The authors advised that surveillance should not be performed in patients who are unlikely to benefit, such as those who were not transplant-eligible with Child Pugh Class C cirrhosis or significant, life-limiting, co-morbidities. Similarly, it was advised against surveillance in low-risk groups such as Hepatitis C and non-alcoholic steatohepatitis patients without cirrhosis given the marginal risk-benefit ratio. Clinicians at NHS Tayside, Scotland, United Kingdom developed an automated 'intelligent liver function testing' (iLFT) algorithm in 2018[142]. Abnormal liver function tests (LFTs) were combined with clinical features, diagnostic criteria, investigation ordering and reporting, and a tracked blood sciences system; the algorithm then generated a diagnosis or descriptor of the abnormality, with fibrosis staging. Of 568 abnormal LFTs, two thirds were managed in primary care, reducing the need for secondary care referrals. The iLFT algorithm is currently being assessed in other United Kingdom centres; this and similar systems could be incorporated into healthcare services during and after the pandemic, minimising secondary care burden. COVID-19 infection has worse outcomes in obese patients. Hence it is important to stress the need for enhanced resources to prevent and treat metabolic syndrome and associated conditions including liver disease[143], and the need strengthen the pathways for recognition and management more than ever.

Provision for remote laboratory investigations and prescribing

Conventionally when patients attend outpatient appointments, they have laboratory investigations and collect prescriptions during their hospital visit. With the increasing use of telemedicine, it is vital that there are safe mechanisms in place to arrange prescriptions and investigations if required. In order to avoid attendance to hospital for tests, organizations can establish blood-hubs outside main hospital sites, preferably multiple, so patients can have laboratory tests done closer to their home and without coming in contact with high risk patient areas[13]. These sites may be able to also provide day-case infusion services including biologics and other intravenous infusions (such as iron infusions) away from main hospitals, *i.e.*, 'hot' sites. In the current digital age, healthcare providers may aim to setup a smart phone apps or a website link for patients to book appointments for tests at convenient times to them which may also avoid overcrowding at these hubs. There should also be a more conventional system (*e.g.*, telephone appointment booking system) in place for patients who may have limited access or knowledge of using online systems or smart-phones. Remote prescribing systems are helpful for patients with chronic liver and GI diseases. A process whereby patient can be posted medication scripts is helpful in institutions that have this in place. Monitoring of medication (*e.g.*, blood tests in patients of immunosuppression agents) is imperative to ensure safe practice. Digital prescribing - whereby the prescriber can send digital prescription to hospital or community pharmacy and these can either by collected by patients or can be posted to them, is an important resource where available. Such E-prescribing provides an auditable trail for governance purposes. These however require robust information technology systems in place and will depend on availability of resources across different parts of the world.

Optimising training opportunities

For endoscopy training there is need to ensure adequate PPE supply so this doesn't hinder trainees' attendance to training lists. Simulation endoscopic training can be utilized in the current pandemic, allowing trainees sufficient hands-on time. Various organizations have already adopted provision of CPD activities and have moved to online platforms. With numerous available online GI/Liver teaching resources, there has also been a boom in modalities for education such as Twitter. FitzPatrick *et al*[144] reviewed how gastroenterology training can thrive during COVID-19[144]. The authors discussed the challenges in learning environment during COVID-19 pandemic

in endoscopy, outpatient and educational settings, and provided proposed solutions. These included adequate PPE supply, use of simulation training along with directed access to limited endoscopic activity for hands-on training opportunities (like involvement in GI bleed management), reinstatement of formal specialty training days, supervisor's proactive discussion with trainee regarding the remote consultation undertaken. Keswani *et al*[145] have reviewed the importance of internet-based learning, simulator training, and adoption of new educational models to maximize training during the pandemic[145]. Digital learning has flourished during COVID-19 crisis and has provided trainees the option of distanced learning.

CONCLUSION

It is clear there has been a major impact of gastroenterology and hepatology training but also service provision due to COVID-19. Healthcare teams throughout the world have attempted to continue care for patients with pre-existing and new presentations of GI and liver conditions; however this provision has been extensively modified and impacted by the pandemic. Now, with new treatments for COVID-19 along with the vaccines, healthcare professionals are moving forward with a hopeful reduction in burden of the disease for patients. This in turn will allow some manoeuvrability with regards to the ongoing required non-COVID-19 service provision. A balance must exist in the fight against COVID-19, but also ensuring ongoing high levels of care to patients with non-COVID-19 diseases. The training of the specialists of tomorrow remains vital, allowing trainees where possible a safe environment to hone their skills gaining relevant expertise but also providing high quality care to patients in the current pandemic. Whilst the COVID-19 pandemic has had a significant impact upon services and patients, novel approaches of service reconfigurations along with optimisation of existing pathways/protocols have been implemented worldwide in an attempt to maintain optimal care for gastroenterology and hepatology patients and service providers.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7649-7660

DOI: 10.3748/wjg.v27.i44.7649

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

MINIREVIEWS

Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells

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Author contributions: Terra SA and Gonçalves AC collected and interpretated the data, drafted the initial manuscript and revised the article critically; Lourenção PLTA and Rodrigues MAM analysed and interpreted the data, drafted the initial manuscript and revised critically; all authors read and approved the final version of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Supported by Supported by São Paulo Research Foundation (FAPESP), No. 2017/26205-9.

Country/Territory of origin: Brazil

Specialty type: Pathology

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

Peer-review report's scientific quality classification

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Abstract

Intestinal neuronal dysplasia type B (IND-B) is a controversial condition among gastrointestinal neuromuscular disorders. Constipation is its most common clinical manifestation in patients. Despite intense scientific research, there are still knowledge gaps regarding the diagnostic criteria for IND-B in the histopathological analysis of rectal biopsies. The guidelines published in the past three decades have directed diagnostic criteria for quantifying the number of ganglion cells in the nervous plexus of the enteric nervous system. However, it is very complex to distinguish numerically what is pathological from what is normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms. Thus, a series of immunohistochemical markers have been proposed to assist in the histopathological analysis of the enteric nervous system. Several of these markers facilitate the identification of other structures of the enteric nervous system, in addition to ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease, providing a view beyond the number of ganglion cells. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

Key Words: Intestinal neuronal dysplasia type B; Constipation; Diagnosis; Gastrointestinal neuromuscular diseases

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

Received: February 12, 2021
Peer-review started: February 12, 2021
First decision: April 18, 2021
Revised: April 26, 2021
Accepted: November 11, 2021
Article in press: November 11, 2021
Published online: November 28, 2021

P-Reviewer: Choi YS
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

Core Tip: There are knowledge gaps regarding the diagnostic criteria for intestinal neuronal dysplasia type B (IND-B) in the histopathological analysis of rectal biopsies. Several immunohistochemical markers have been proposed to identify other structures of the enteric nervous system, beyond the ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

Citation: Terra SA, Gonçalves AC, Lourenção PLTA, Rodrigues MAM. Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells. *World J Gastroenterol* 2021; 27(44): 7649-7660
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7649.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7649

INTRODUCTION

The histopathological diagnosis of colon diseases presenting with severe constipation in childhood has been a challenge in recent decades. Hirschsprung's disease (HD), the best known among the gastrointestinal neuromuscular diseases, is defined by the absence of ganglion cells in the submucosal and myenteric plexuses of the enteric nervous system (ENS). Intestinal neuronal dysplasia type B (IND-B) is characterized by hyperplasia of the submucosal nerve plexuses[1-4] (Table 1).

IND-B was first described in 1970, when Nezelof *et al*[5] reported three cases of megacolon associated with hyperplasia of the myenteric nervous plexus. A year later, Meier-Ruge[6] defined it as a condition usually associated with low intestinal obstruction that could simulate HD but showed distinct histopathological characteristics such as hyperplasia of the nerve plexuses and increased activity of the enzyme acetylcholinesterase (AChE) in the parasympathetic nerve fibers of the lamina propria of the mucosa.

Almost 50 years have passed and, despite the intense scientific research conducted during this period, there are still uncertainties, including IND-B etiopathogenesis, diagnostic criteria, and therapeutic possibilities. Thus, IND-B is currently considered as a controversial condition among the differential diagnoses of organic causes of intestinal constipation[7-10].

Most patients with IND-B are children showing chronic constipation. The main symptoms are very similar to those observed in patients with HD. Delay in the meconium passage, abdominal distention, vomiting, and difficulty in eating can occur during the first days of life[7,9]. A portion of these patients can show symptoms throughout their lives, evolving to severe constipation refractory to different types of clinical treatment[7,9,11,12]. Severe symptoms such as episodes of enterocolitis, acute intestinal obstruction, volvulus, and intussusception, although rare, are possible complications in different age groups[12-14]. Recent studies have highlighted the growing number of cases in adults, some showing symptoms of constipation since childhood, and others with late onset of symptoms[15-19]. The oldest patient reported in the literature with a diagnosis of IND-B was 71 years old. This patient showed symptoms since childhood, with severe constipation for more than 60 years, including several hospitalizations and surgeries during this period[19].

Two therapeutic modalities have been used in patients with IND-B: conservative clinical treatment and surgical treatment[9,20]. Conservative treatment is based on changes in diet, laxatives, and enemas in cases of fecal retention[7,20,21]. Surgical treatment can be performed through sphincterotomy, extensive surgical resection, or temporary colostomy[22-25]. However, the outcomes obtained using different treatment modalities show conflicting results[11,20,21]. Currently, the most accepted trend is that patients diagnosed with IND-B who have no complications must receive conservative treatment[1,3,10,20].

Table 1 Comparison between Hirschsprung's disease and intestinal neuronal dysplasia type B regarding the main clinical and histopathological aspects

	HD	IND-B
Histopathological findings on rectal biopsies	Absence of ganglion cells in the submucosal and myenteric plexuses	Hyperplasia of the submucosal nerve plexuses
Clinical Picture	Neonatal bowel obstruction or severe constipation	Neonatal bowel obstruction or severe constipation
Treatment	Surgical (colorectal pull-through)	Diet, laxatives, enemas or surgical (if complications)

HD: Hirschsprung's disease; IND-B: Intestinal neuronal dysplasia type B.

CONTROVERSIES REGARDING THE EXISTENCE OF IND-B

One of the main controversies in the understanding of IND-B is related to the existence of a cause-effect relationship between the histopathological findings and clinical symptoms. In most cases, a diagnosis of IND-B is based on the histopathological analysis of rectal biopsies in patients with constipation that is usually refractory to clinical treatment. A minority of patients have acute intestinal obstruction or enterocolitis[7,9]. In contrast, histopathological alterations compatible with the diagnosis of IND-B were observed in the colon segments of 36 asymptomatic children[26]. Other studies have failed to demonstrate a direct correlation among histopathological findings, clinical symptoms, and radiological and manometric changes[27,28]. Given these controversies, some authors show skepticism concerning the definition of IND-B as a true clinical condition, preferring to define it as a histopathological alteration of the ENS, which may not present symptoms[7,10,29].

In a recent review on the subject, Kapur and Reyes-Mugica[10] concluded that IND-B remains an undefined histopathological phenotype of uncertain clinical relevance, and that it is imprudent to make clinical decisions based on this histopathological diagnosis, while claiming that this type of histopathological finding may represent deviations from normality. Moreover, these authors criticize the most recent scientific publications that investigated patients with this diagnosis, arguing that they have perpetuated the debate on dubious and controversial concepts. However, in medical practice, we continue to find children with severe constipation or intestinal obstructions who undergo diagnostic investigation for HD and, in the histopathological analysis of rectal biopsies, present ganglion cells hyperplasia in the nervous plexuses of the submucosa. Schmittenbecher *et al*[11] considered that these morphological changes were compatible with a diagnosis of IND-B, which should be considered as a distinct clinical condition, since the symptoms presented by the patients were evident and often persisted for several years, directly influencing their quality of life. The authors argued that patients with IND-B should be treated in a specific manner and highlighted the need to develop specific treatment algorithms. The majority of experts who participated in the Fourth International Symposium on Hirschsprung's disease and related neurocristopathies in Genova, Italy, in 2004 and in a recent survey by the European Association of Pediatric Surgeons, consider IND-B to be a clinicopathological condition, justifying the need for further studies on this disease[30,31].

LACK OF DIAGNOSTIC CRITERIA

The lack of well-established criteria for the histopathological diagnosis of IND-B can be considered as the basis for many of the uncertainties described. To understand any disease, it is mandatory to know how to diagnose it. In the case of IND-B, the morphological criteria for histopathological diagnosis have been modified over the years, making comparisons between studies difficult and increasing the controversies and doubts concerning its existence[7,32,33]. Hyperplasia of the nervous plexuses of the ENS is a morphological finding that defines IND-B (Figure 1), but it is differently characterized by varying criteria[6,9].

Based on the 1990 Frankfurt Consensus discussions, Borchard *et al*[32] established morphological criteria for the histopathological diagnosis of IND-B by using biopsies

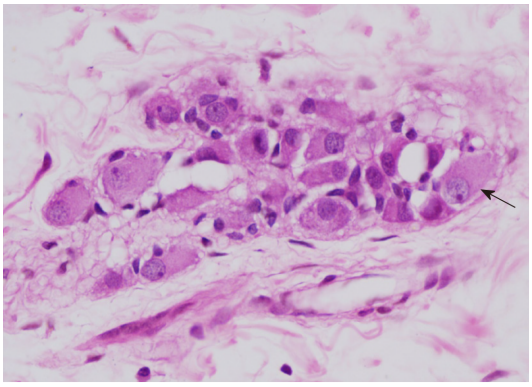

Figure 1 Intestinal neuronal dysplasia type B. Submucosal nerve plexus with hyperganglionosis: giant ganglion. Ganglion cell (arrow) (H&E, 400 ×).

of the rectal wall (Table 2). Since then, these criteria have been widely used, both in clinical practice, follow-up studies and in investigations into the pathophysiology and etiopathogenesis of IND-B[24,27,34].

New diagnostic criteria were proposed in the 1990s, highlighting the need to identify giant ganglia in the submucosa, characterized by the presence of a minimum number of ganglion cells, ranging from six to more than 10 per ganglion[35,36]. In 2004, Meier-Ruge *et al*[33] proposed a quantitative criterion for the histopathological diagnosis of IND-B (Table 2). Since this diagnosis depends on quantitative data, which can be compromised by technical variables, the entire laboratory process must be highly standardized. The biopsy must be adequate to obtain a sufficient quantity of submucosal tissue for analysis, and the frozen sections must be 15-μm thick and analyzed for a specific histochemical panel composed of lactate dehydrogenase, succinyl dehydrogenase, and nitric oxide (NO) synthase[1,33]. Although these criteria have been accepted by the scientific community, they were used in few case reports [37]. The need for fresh material and specific histochemical stains, the availability of which is restricted to certain centers, is a major limitation for diagnosis[9,38,39]. It is also uncertain whether these criteria can be applied to 5-μm thick histological sections from paraffin-embedded material used for conventional histological analysis with hematoxylin and eosin (H&E) and immunohistochemical methods[4,39]. Taguchi *et al* [37] analyzed data from 161 Japanese gastroenterological and pediatric surgery institutions from January 2000 to December 2009 and found 355 patients with HD and allied disorders. Of these, 13 patients were diagnosed with IND-B based on the histopathological criteria commonly used, but only four met the quantitative criteria proposed by Meier-Ruge *et al*[33]. Terra *et al*[39] analyzed surgical specimens from 29 patients diagnosed with IND-B, previously established by the criteria of the Frankfurt Consensus[32], in the histological sections processed for conventional histology by H&E. Only one patient met the numerical diagnostic criteria proposed by Meier-Ruge *et al*[33]. The authors concluded that the recommended quantitative criteria for 15-μm cuts stained by specific histochemical panels have limited applicability when transposed to conventional histological analysis.

Thus, the changes proposed in the last three decades have oriented the diagnostic criteria for quantifying the number of ganglion cells in the nervous plexuses of the ENS[33,35,36]. However, it is very complex to numerically distinguish the pathological from the normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms[1,10,40]. Obtaining intestinal samples for use as controls has ethical limitations because of the need for invasive procedures to obtain tissue samples. In some studies, the control group was composed of intestinal samples from patients with congenital intestinal malformations such as anorectal abnormalities, which cannot be considered healthy controls[41]. In addition, the variation in the number of ganglion cells related to age, especially during the first year of life, leads to the need for age-matched controls, making it difficult to develop an appropriate control group[26]. The thickness of the histological section, the staining techniques, and the methods of histopathological analysis directly influence the number of ganglion cells identified in the count[38,39]. For all these reasons, the quantitative criteria seem to have contributed to more uncertainty rather than greater objectivity for the diagnosis of IND-B. There are important differences regarding the minimum number of ganglion cells and nerve ganglia, questions concerning the different methods of histopathological analysis, and the possibility of a minimum age

Table 2 Summary of the histopathological criteria for the diagnosis of intestinal neuronal dysplasia type B

Diagnostic criteria	Definition	
Frankfurt Consensus[32], 1990	Mandatory criteria	Submucosal plexus hyperplasia
		Increased activity of the AChE enzyme in nerve fibers around submucosal blood vessels
	Complementary criteria	Neuronal heterotopy
		Increased AChE activity in the lamina mucosa
Meier-Ruge *et al*[33], 2004	Quantitative criteria	At least 20% of giant nerve ganglia[1] in the submucosa, in 25 submucosal ganglia
	Age criteria	Patients must be older than 1 yr

[1]Giant nerve ganglia: A ganglion with more than eight ganglion cells.
AChE: Acetylcholinesterase.

for diagnosing this disease[39].

UNCERTAINTIES AND RECENT ADVANCES

Pathophysiology and etiopathogenesis are poorly defined aspects of IND-B[7,30]. Some authors consider these aspects as part of the normal development of the ENS[3]. As age advances, there is an increase in the size of ganglion cells and a decrease in their number per plexus[26,36,40]. These findings can be related to a physiological process of maturation, which involves the apoptosis of components of the ENS[1,33]. Schimpl *et al*[21] retrospectively analyzed 105 patients with IND-B: 60.95% of patients were less than 6 mo old, 30.47% were between 6 and 12 mo old, and only 8.57% were more than 1 year old. This percentage distribution, which decreases with age, suggests a maturation process of the ENS or some abnormality in the development of the intestinal nervous system, with spontaneous normalization with advancing age.

Other studies have raised the possibility that IND-B represents an adaptive response of the ENS, secondary to obstructive or inflammatory bowel phenomena that occur in the fetal, perinatal, or postnatal period. There are reports of morphological findings suggestive of IND-B in the intestinal areas immediately above intestinal atresia, rectal mucosa prolapse, imperforate anus, and necrotizing enterocolitis[27,42,43]. These secondary responses to obstructions or inflammations have been studied experimentally, and the results are conflicting[44,45]. Using a model of partial intestinal colon obstruction in rats, Moore *et al*[44] showed a decrease in the number of ganglion cells compared to that in the control group, explained by an increase in colon diameter due to the distention of its wall. Using a model of chronic intestinal obstruction in adult rats, Gálvez *et al*[45] identified histopathological changes compatible with IND-B, with an increase in the number of ganglion cells.

Recent studies have shown that IND-B can originate from genetic alterations, which directly influence the embryological development of tissues derived from the neural crest. Angelini *et al*[41] showed significantly unregulated expression of microRNAs in the submucosal and muscular layers of the colon and peripheral blood of patients with IND-B. The molecular pathways biologically regulated by these microRNAs (axon guidance, nerve growth factor signaling, neural cell adhesion molecule (NCAM) signaling for neurite outgrowth, neuronal system, and apoptosis) show activities related to the ENS and, therefore, can be related to the pathogenesis of IND-B. Liu *et al*[46] showed a decrease in the methylation levels of locus 32 on the *Sox10* promoter gene in the peripheral blood of patients with IND-B. There was a negative correlation between these levels and Sox10 expression in the colons of these patients. These changes can contribute to the regulation of the number of intestinal glial cells and the maturation of neurons in the ENS[46].

In addition, reports on familial occurrence of IND-B and its association with other intestinal and extraintestinal diseases reinforce the theory that IND-B has a primary, genetically determined origin[34,47-49]. Among these associations, HD, incomplete intestinal rotation, and multiple endocrine neoplasia type 2 (NEM 2) are the most important[23,28].

Histopathological findings compatible with IND-B in segments proximal to areas of aganglionosis are not uncommon and have been considered as a possible cause of the persistence of obstructive symptoms in patients undergoing surgical treatment for HD.

This association was first reported in the 1970s[50]. Since then, the association between IND-B and HD has been described with rates ranging from 6% to 75% of HD cases[38,51,52]. In these cases, the presence of colonic segments with IND-B can be explained both by primary embryological alteration of the ENS, which gives rise to neuropathies, and by secondary adaptation to distal intestinal obstruction, caused by the spastic aganglionic intestinal segment[22,53].

IMMUNOHISTOCHEMISTRY AND LOOKING BEYOND THE NUMBER OF GANGLION CELLS

Over the past two decades, a series of immunohistochemical markers have been proposed to assist in histopathological analysis of the ENS[2,4,54-57]. The use of calretinin, well established in HD, is the primary example of how an immunohistochemical method can contribute substantially to the diagnosis of enteric neuropathy. The absence of immunohistochemical expression of calretinin in colorectal aganglionic segments has been routinely used and is part of the main guidelines for HD diagnostic management[58,59]. Terra *et al*[39] identified the expression of calretinin in ganglion cells and mucosal nerve fibers in 29 patients with IND-B. Immunoexpression of this marker was observed in ectopic neurons in the lamina propria and in the muscularis mucosa (Figure 2), which was not previously identified using standard histology (H&E).

Several studies have assessed the role of immunohistochemical methods both in investigating aspects related to etiopathogenesis and in improving the histopathological diagnosis of IND-B. These studies[39,46,54,60-71] are summarized in the table presented in Supplementary Table 1. The pattern of immunoexpression of the main markers described in patients with IND-B is shown in Table 3.

Bosman *et al*[66] identified an increase in the number of ganglion cells, with positive expression of NO synthase in the intestine of patients with IND-B compared to that in the control group. This enzyme is responsible for the synthesis of NO, a neurotransmitter capable of inducing smooth muscle relaxation that may be responsible for the clinical symptoms of intestinal pseudo-obstruction presented by patients.

Yamataka *et al*[62] showed a reduction in c-kit and 171B5 (synaptophysin) expression in the muscle layers, with the exception of the myenteric plexuses, in the intestine of patients with IND-B compared to that in the control group. The c-kit protein is expressed in the interstitial cells of Cajal and is considered to be a regulator of gastrointestinal tract motor activity[72,73]. Synaptophysin is expressed in the protein membrane of synaptic vesicles and is present during the neurotransmission of the central and peripheral nervous systems, including the ENS. The reduction in the expression of these two markers raises the hypothesis of the role of interstitial cells of Cajal in the pathophysiology of IND-B[62].

Kobayashi *et al*[63] reported that the markers NCAM, growth-associated protein 43 (GAP43), and synaptophysin showed absence of expression or markedly decreased expression in the muscular layers of the intestine of patients with IND-B compared to that in the control group. No change was observed in the expression of these markers in the nervous plexus of the ENS. NCAM is a surface glycoprotein that is important for the interaction between neurons and muscle cells during synaptogenesis and is considered as a marker of the neuromuscular junction[74]. GAP43 is a marker associated with neuron development and regeneration during axonal growth and is found in high concentrations in presynaptic areas[63]. These changes in the expression patterns of these markers led the authors to propose the hypothesis that patients with IND-B presented defects in the innervation of neuromuscular junctions, which would explain the changes in colonic motility. However, Nogueira *et al*[65] were unable to reproduce this pattern of NCAM and synaptophysin expression, weakening this hypothesis.

Kobayashi *et al*[64] showed an increase in the number of mast cells, identified using immunohistochemical methods, in the intestinal segments of patients with IND-B and in the aganglionic segments of HD patients compared to that in the normoganglionic segments of HD patients and individuals in the control group. Mast cells were also identified by the marker nerve growth factor (NGF) close to the nerve ganglia in patients with IND-B and close to the hypertrophied nerve trunks in the aganglionic segments of HD. Based on these observations, the authors suggested the possible participation of mast cells in the pathogenesis of IND-B and HD.

Table 3 Immunohistochemical markers for the diagnosis of intestinal neuronal dysplasia type B

Immunohistochemical marker	Immunoexpression pattern
Bcl-2[54]	Positive expression in immature and mature ganglion cells
Calretinin[39,54]	Positive expression in mature ganglion cells in submucosal and myenteric plexuses
	Positive expression in intrinsic nerve fibers and ectopic neurons in the mucosa
c-kit[60-62]	Positive expression in Cajal cells in muscle layers and myenteric nerve plexuses
GAP43[63]	Absence of expression in muscularis mucosae and/or in the circular muscular and/or longitudinal muscular
	Positive expression in nerve plexuses
Hu C/D[38]	Positive expression in mature ganglion cells in submucosal and myenteric plexuses
NGF (mast cells)[64]	Expression of mast cells close to ganglia
NCAM (CD56)[60,61,63,65]	Positive expression in neurons of the muscular layers
	Variable expression in nerve fibers in muscle layers and mucosa
NO synthase[66]	Positive expression in ganglion cells
NSE[61]	Positive expression in nerve fibers in the muscle layers and mucosa
Peripherin[66,67]	Positive expression in ganglion cells
PGP9.5[60,68]	Positive expression in enlarged nerve trunks, hyperplastic nerve ganglia, and heterotopic nerve cells
	Positive expression in neurons of the inner intestinal muscle layer
PTEN[69]	Reduced expression in submucosal and myenteric nerve plexuses
RET[54,66]	Positive expression in mature and immature ganglion cells
S100[60,61,66]	Positive expression in ganglion cells
	Positive expression in nerve fibers in the muscular layers and mucosa
Synaptophysin[60,62,63,65,66,70]	Positive expression in synaptic vesicles in plexuses and nerve fibers
SMA (1a4)[61,71]	Positive expression in muscle fibers from muscular layers and muscularis mucosa
Sox10[46]	Positive expression in glial and ganglion cells in nerve plexuses

Bcl-2: B-cell lymphoma 2; c-kit: Transmembrane tyrosine kinase receptor; GAP43: Growth associated protein 43; Hu/CD: RNA binding protein Hu (HuC and HuD) - also referred to as type-1 anti-neuronal nuclear antibodies (ANNA-1); NGF: Nerve growth factor; NCAM: Neural cell adhesion molecule; NO: Nitric oxide; NSE: Neuron-specific enolase; PGP9.5: Protein gene product 9.5; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; RET: Rearranged during transfection - immunohistochemical detection of RET proto-oncogene; SMA: Smooth muscle antibody; Sox10: SRY-box transcription factor 10.

O'Donnell and Puri[69] evaluated the immunohistochemical expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in patients with IND-B. PTEN is a protein involved in cell proliferation, survival, and migration and has a potential modulatory role in neurogenesis and synaptic plasticity. The authors showed significantly reduced expression of this marker in the myenteric and submucosal plexuses of the intestine of patients with IND-B, absence of PTEN expression in the aganglionic segments of HD patients, and strongly positive expression in ganglionic intestinal segments of HD patients and individuals in the control group. Based on these results, the authors suggested that the giant ganglia with immature cells, present in IND-B, might be associated with a reduction in PTEN level, which might be responsible for the lack of control in neuronal growth and proliferation.

Some markers have demonstrated the potential to assist in neuron counting, which is fundamental for the quantitative diagnosis of IND-B. Geramizadeh *et al*[60] evaluated the immunohistochemical expression of the protein gene product 9.5 (PGP9.5), a specific cytoplasmic marker of the nervous system, S100, a nerve cell nucleus and cytoplasm marker, c-kit, synaptophysin, and NCAM to count nerve cells in the muscle layers and myenteric plexuses of the distal colon of patients with HD,

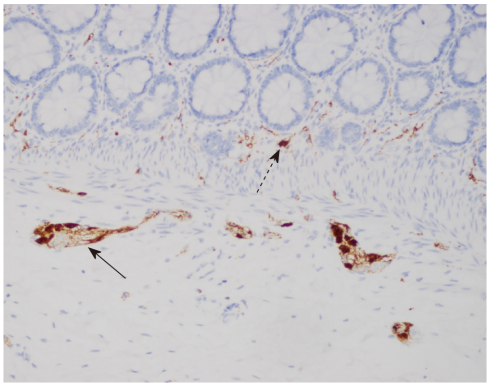

Figure 2 Calretinin immunohistochemistry in intestinal neuronal dysplasia type B. Positive nuclear calretinin staining in neurons from a submucosal nerve plexus (arrow). Heterotopic neuron in the muscularis mucosa (dotted arrow) (200 ×).

IND-B, and the control group. The authors showed a significantly higher number of nerve cells, stained by these immunohistochemical methods, in the muscle layers and/or the myenteric plexuses in patients with IND-B than in HD patients. However, this difference was not significant when patients with IND-B were compared to those in the control group. Kim *et al*[61] showed no significant differences in the counts of interstitial cells of Cajal (c-kit immunopositive) in muscle layers and myenteric plexuses in patients with IND-B compared to those in patients with other types of intestinal pseudo-obstructions.

Swaminathan *et al*[38] evaluated the use of the pan-neuronal immunohistochemical marker Hu C/D in the quantitative analysis of ganglion cells in colonic segments with IND-like submucosal ganglion cell hyperplasia at the proximal margins of HD resections. This marker detects neuronal cell bodies, facilitating the recognition of neurons, and has been used as a neuronal marker for the central and peripheral nervous systems[75]. By counting the nuclei of ganglion cells immunoexpressing Hu, it was possible to develop and validate a quantitative histopathological criterion for the diagnosis of giant ganglia, defined by the presence of at least seven ganglion cells [38].

Wang *et al*[54] determined the number of ganglion cells and the area of myenteric nerve plexuses using immunohistochemical expression of the RET protein, which is encoded by the *RET* gene and is related to cell growth and differentiation, and of B-cell lymphoma 2 (Bcl-2), a protein encoded by the homonymous gene located on chromosome 18q21, which plays a role in the maintenance of cell survival. The authors identified a significantly higher number of ganglion cells and a larger myenteric plexus area in the colon of patients with IND-B than in that of the control group.

CONCLUSION

IND-B remains a controversial clinicopathological condition, with a poorly understood etiopathogenesis and a series of challenges related to its diagnosis. There is no consensus regarding the criteria used by different centers for the histopathological diagnosis of IND-B. Despite these uncertainties, patients continue to show severe constipation or intestinal obstruction, associated with histopathological alterations compatible with this morphological phenotype, which fully justifies all the efforts directed toward new diagnostic strategies. Only the correct diagnosis of these patients can lead to more effective treatment and better prognosis. In this regard, immunohistochemical techniques have identified other ENS structures, in addition to the ganglion cells of the submucosa and muscular nerve plexuses. These structures may be potential candidates for biomarkers in the histopathological diagnosis of IND-B, expanding the horizon beyond counting the number of ganglion cells.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7661-7668

DOI: 10.3748/wjg.v27.i44.7661

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

MINIREVIEWS

Treatment of *Helicobacter pylori* infection in the presence of penicillin allergy

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Author contributions: Dutta AK and Phull PS contributed equally to this work, jointly undertook the literature review; Phull PS had the idea for the review; Dutta AK wrote the first draft of the manuscript, which was revised by Phull PS; all authors have read and approved the final manuscript.

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

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

Therapy of *Helicobacter pylori* (*H.pylori*) requires a combination of antibiotics together with an acid suppressing agent; most treatment regimens include Amoxicillin as one of the antibiotics, which is an important constituent as resistance to it is low. However, allergies to the penicillin group of antibiotics are not uncommon, and treating *H.pylori* infection in such individuals can be challenging due to the restricted choice of regimens. The aim of this review is to summarise the evidence for therapeutic options in patients with *H.pylori* infection and penicillin allergy. A literature search was conducted in PubMed for English language publications using the key words 'Helicobacter' and 'treatment' or 'therapy' and 'penicillin' or 'beta-lactam' and 'allergy' or 'anaphylaxis'. Eighteen studies were identified that specifically evaluated *H.pylori* treatment success in penicillin allergic patients. The number of subjects in most of them was low and many were retrospective, uncontrolled, single cohort studies. The most effective option for first-line treatment appears to be Bismuth-based quadruple therapy for 10-14 d. The evidence supports second-line treatment with Levoflaxacin-based triple therapy for 10 d. Patients with persistent *H.pylori* infection after 2 treatment courses should be considered for testing to confirm penicillin allergy. Further treatment should be guided by the results of *H.pylori* culture and sensitivity testing.

Key Words: *Helicobacter pylori*; Infection; Treatment; Penicillin-allergy; Stomach; Duodenum

Received: April 12, 2021
Peer-review started: April 12, 2021
First decision: June 3, 2021
Revised: June 17, 2021
Accepted: November 10, 2021
Article in press: November 10, 2021
Published online: November 28, 2021

P-Reviewer: Cheng H, El-Bendary M, Ma H
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

Core Tip: Penicillin allergy is a not uncommon occurrence and treating *Helicobacter pylori* infection in such individuals can be challenging. This review highlights the lack of high-quality studies to help guide management strategies. Recommendations have been made based on the limited data, but it would be important to monitor the success of treatment regimens and use what can be demonstrated to be effective locally.

Citation: Dutta AK, Phull PS. Treatment of *Helicobacter pylori* infection in the presence of penicillin allergy. *World J Gastroenterol* 2021; 27(44): 7661-7668
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7661.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7661

INTRODUCTION

Infection with *Helicobacter pylori* (*H.pylori*) is prevalent worldwide with about half of world's population estimated to be affected by this gram negative spiral bacterium[1]. The organism is causally implicated in the pathogenesis of peptic ulcer disease[2] and gastric adenocarcinoma[3]. Guidelines for the management of *H.pylori* infection have been published by a number of national societies and organisations[4-7].

Therapy of *H.pylori* requires a combination of antibiotics together with an acid suppressing agent (proton-pump inhibitor, PPI); most treatment regimens include Amoxicillin as one of the antibiotics, which is a particularly important constituent as resistance to it is low[8]. However, allergies to the penicillin group of antibiotics are reported in 5% to 15% of patients in developed countries[9] and, consequently, the treatment options in individuals allergic to penicillin are significantly restricted.

In this review we summarise the available evidence for therapeutic options in patients with *H.pylori* infection and penicillin allergy.

LITERATURE SEARCH

A literature search was conducted in PubMed using the key words 'Helicobacter' and 'treatment' or 'therapy' and 'penicillin' or 'beta-lactam' and 'allergy' or 'anaphylaxis' for English language publications from database commencement until January 31, 2021. Of the 77 publications identified, 18 studies were included in the review (48 were excluded as not relevant, and 11 were review articles)[10-27].

EVIDENCE FOR TREATMENT OF *H.PYLORI* INFECTION IN THE PRESENCE OF PENICILLIN ALLERGY

Data from studies specifically targeting penicillin allergic patients (Table 1). Considering the large volume of publications on *H.pylori* therapy, there is relatively little data on treatment of this bacterium in penicillin allergic individuals. A summary of data available from the 18 identified studies is shown in the Table. It should be noted that the number of subjects included in most of them is quite low and many are retrospective, uncontrolled, single cohort studies. All results discussed below are presented on an intention-to-treat basis.

First line therapy

Dual therapy: Prach *et al*[10] reported 100% treatment success with a 14 d combination of Omeprazole and Clarithromycin; however, this was only in 3 patients.

Triple therapy: The success rate with the 7 d PPI-Clarithromycin-Metronidazole regimen, has been reported as 50%-83.3% in retrospective studies[20,21] and 54-58% in prospective studies[11,14,18]. A longer 14 d regimen also resulted in a low success rate at 63.6%[23]. The European Registry on *H.pylori* management (Hp-EuReg) has provided the largest experience of treatment in penicillin allergic patients[25]. Although drug dose, frequency and duration details were not provided, the PPI-Clarithromycin-Metronidazole regimen achieved 69% success rate. Two studies from

Table 1 Published studies of *Helicobacter pylori* eradication therapy in patients allergic to penicillin

Ref.	Yr	Country	Study type	Treatment details	*n*	Success rate (PP, %)	Success rate (ITT, %)
Prach *et al*[10]	1998	United Kingdom	Prospective,single cohort	*1st line* O 20 mg b.d., C 500 mg t.d.s, 14 ds	3	100;	100;
Gisbert *et al*[11]	2005	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* RBC 400 mg b.d., T 500 mg q.d.s., M 250 mg q.d.s., 7 d; *3rd line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; *4th line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 ds	12; 17; 9; 2	64; 53; 17; 100	58; 47; 11; 100
Rodriguez-Torres *et al*[12]	2005	Puerto Rico	Prospective, single cohort	*1st line* E 40 mg q.d.s., T 500 mg q.d.s., M 500 mg q.d.s.,10 d; *2nd line* E 40 mg q.d.s., T 500 mg q.d.s., M 500 mg q.d.s.,10 d	17; 3	NA; NA	85; 100
Matsushima *et al*[13]	2006	Japan	Retrospective, single cohort	*1st line* PPI o.d., T 500 mg b.d., M 250 mg b.d., 7-14 d	5	100	80
Gisbert *et al*[14]	2010	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d	50; 15	55; 73	54; 73
Tay *et al*[15]	2012	Australia	Prospective, single cohort	*2nd line* R 20 mg t.d.s., B 240 mg q.d.s., RIF 150 mg b.d., CF 500 mg b.d., 10 d	69	94.2	94.2
Liang *et al*[16]	2013	China	Prospective, randomised	*2nd line* 109 pen allergic overall but results reported for whole group including non-allergic; L 30 mg b.d., B 220 mg b.d., T 500 mg t.d.s., F 100 mg t.d.s., 14 d; L 30 mg b.d., B 220 mg b.d., T 500 mg q.d.s., M 400 mg q.d.s., 14 d	108; 107	96.1; 93.1	91.7; 87.9
Furuta *et al*[17]	2014	Japan	Retrospective, single cohort	*1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d; *2nd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d; *3rd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *3rd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d	7; 4; 9; 3; 3; 2	100; 100; 100; 100; 100; 100	100; 100; 100; 100; 100; 100
Gisbert *et al*[18]	2015	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *3rd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *4th line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; *3rd line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; 4th line O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *3rd line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *1st line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d	112; 24; 3; 2; 7; 2; 50; 3; 50; 14	59; 38; 50; 0; 20; 100; 73; 100; 75; 64;	57; 37; 33; 50; 14; 100; 64; 100; 74; 64;
Mori *et al*[19]	2017	Japan	Prospective, single cohort	*1st line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d; *2nd line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d; *3rd line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d	33; 19; 5	100; 84.2; 40	100; 84.2; 40
Ono *et al*[20]	2017	Japan	Retrospective,single cohort	*1st line* PPI b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *1st line* V 20 mg b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *1st line* V 20 mg b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd line* PPI b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *2nd line* V 20 mg b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *2nd line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd line* V 20 mg b.d., SF 100 mg b.d., M 250 mg b.d., 7 d	10; 13; 20; 14; 3; 1; 24; 3	55.6; 92.3; 100; 100; 33.3; 100; 100; 66.7	50; 92.3; 100; 92.9; 33.3; 100; 100; 66.7
Sue *et al*[21]	2017	Japan	Prospective& retrospective, single cohort	*1st line* V 20 mg b.d., C 200 or 400 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d, C 200 or 400 mg b.d, M 750 mg b.d., 7 d	20; 30	100; 86.2	100; 83.3
Osumi *et al*[22]	2017	Japan	Prospective, single cohort	*1st line* R 20 mg b.d, Mi 100 mg b.d, M 250 mg b.d., 7 d	5	100	100
Long *et al*[23]	2018	China	Prospective, randomised	*1st line* E 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d; *1st line* E 20 mg b.d., B 600 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d	33; 33	70; 96	63.6; 84.8

Song *et al*[24]	2019	China	Prospective, single cohort	*1st line* E 20 mg b.d, B 220 mg b.d., LF 500 mg o.d., Cef 500 mg b.d., 14 d	152	90.1	85.5
Nyssen *et al* [25]	2020	Europe	Retrospective,multi-centre registry	*1st line* PPI, C, M; *1st line* PPI, C, LF; *1st line* PPI, B, T, M; *2nd line* PPI, C, LF; *2nd line* PPI, M, LF; *2nd line* PPI, B, T, M; *3rd line* PPI, B, T, M; *3rd line* PPI, B, C, LF; *3rd line* PPI, C, LF *(NB drug dose, frequency and duration not specified)*	285; 54; 250; 20; 13; 70; 18; 1; 2	69; 82; 92; 73.7; 76.5; 81.8; 77.8; 100; 50	69; 80; 91; 75; 76.5; 78.3; 77.8; 100; 50
Luo *et al*[26]	2020	China	Prospective, single cohort	*1st & 2nd line* E 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d; *1st & 2nd line* E20 mg b.d., B 220 mg b.d., C 500 mg b.d., M 400 mg q.d.s., 14 d; *1st & 2nd line* E 20 mg b.d., LF 500 mg o.d., M 400 mg b.d., 14 d; *1st & 2nd line* E 20 mg b.d., B 220 mg b.d, LF 500 mg o.d., M 400 mg q.d.s., 14 d; *1st & 2nd line* E 20 mg b.d., T 500 mg q.d.s., M 400 mg b.d., 14 d; *1st & 2nd line* E 20 mg b.d., B 220 mg b.d, T 500 mg q.d.s., M 400 mg q.d.s., 14 d	5; 22; 1; 10; 2; 72	100; 94.1; 100; 100; 100; 100	100; 81.8; 100; 80; 100; 97.2
Sue *et al*[27]	2021	Japan	Prospective, single cohort	*2nd line* V 20 mg b.d,, SF 100 mg b.d., M 250 mg b.d., 7 d	17	88.2	88.2

PP: Per protocol analysis; ITT: Intention to treat analysis; B: Bismuth compound; C: Clarithromycin; Cef: Cefuroxime; CF: Ciprofloxacin; E: Esomeprazole F: Furazolidone; LF: Levofloxacin; M: Metronidazole; Mi: minocycline O: Omeprazole; PPI: proton pump inhibitor; R: Rabeprazole; RBC: ranitidine bismuth subcitrate; RIF: Rifabutin; SF: Sitafloxacin; T: Tetracycline; V: Vonoprazan.

Japan, have shown higher success rates (92.3%-100%) for this 7 d triple therapy when combined with Vonoprazan (a potassium-competitive blocker that inhibits gastric H+K+-ATPase) instead of a PPI[20,21].

In a prospective study, Rodriguez-Torres *et al* reported a success rate of 85% with a 10 d triple therapy combining Esomeprazole, Tetracycline and Metronidazole[12]. A small retrospective study from Japan also reported a similar success rate of 80% when this regimen was used for 7-14 ds[13]. Osumi *et al* achieved a 100% success rate using a modified 7 d regimen, substituting Minocycline for Tetracycline in a small study of 5 patients[22].

Levofloxacin in combination with Clarithromycin and PPI has been reported to achieve an 80% success rate[25]. Recent studies from Japan have evaluated treatment regimens utilising the fluoroquinolone, Sitafloxacin, which has a lower minimum inhibitory concentration for *H.pylori* than Levofloxacin and is effective in strains with the gyrA mutation, which denotes resistance to Levofloxacin[19]. Remarkably high success rates of 100% were reported for 7-14 d treatment regimens combining Sitafloxacin with Metronidazole and PPI, in two retrospective[17,20] and one prospective study[19].

Quadruple therapy: Retrospective data has demonstrated a 91% success rate for the PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[25]. Three prospective studies have reported success rates of 74% with a 10 d PPI-Bismuth-Tetracycline-Metronidazole combination[18], 84.8% with a 14 d PPI-Bismuth-Clarithromycin-Metronidazole regimen[23] and 85.5% with a 14 d PPI-Bismuth-Levofloxacin-Cefuroxime treatment[24].

Second line therapy

In view of the attrition of successfully treated patients with each course of treatment, patient numbers for studies evaluating second line therapies tend to be low, often in single digits[12,17,20].

Triple therapy: Gisbert *et al*[14,18] have treated a relatively large number of patients with a 10 d combination of PPI-Clarithromycin-Levofloxacin, demonstrating success rates of 64%-73%. Levofloxacin based triple therapy using Clarithromycin or Metronidazole appears to achieve similar success rates, of 75% and 76.5%, respectively [25]. Sitafloxacin-based triple therapy has shown success rates of 100% in 2 small retrospective studies evaluating a 7 d regimen[17,20], whilst a prospective study investigating a 10 d treatment course reported a lower success rate of 84.2%[19]. Sue *et al*[27] demonstrated a success rate of 88.2% in a prospective study of a 7 d Sitafloxacin regimen using Vonoprazan instead of a PPI.

Quadruple therapy: An early study from Spain reported a low success rate of 47% using a 7 d regimen of Ranitidine Bismuth citrate-Tetracycline-Metronidazole, which has been considered as quadruple therapy due to an acid-suppressing agent and bismuth being combined into one tablet[11]. The same group of investigators also reported a low success rate of 37% for 10 d PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[18]. However, the European Registry has demonstrated a success rate of 78.3% for this regimen[25]. In a large prospective study, Liang *et al*[16] included 109 penicillin allergic patients randomised to 2 wk quadruple therapy with either PPI-Bismuth-Tetracycline-Metronidazole or PPI-Bismuth-Tetracycline-Furazolidine; success rates were 87.9% and 91.7%, with no difference between penicillin allergic and non-allergic patients[16].

A relatively large study from Australia reported on 69 patients with penicillin allergy, who had all failed prior therapy with PPI-Clarithromycin-Metronidazole. Treatment with a 10 d regimen of PPI-Bismuth subcitrate-Rifabutin-Ciprofloxacin achieved a success rate of 94.2%[15].

Luo *et al*[26] prospectively evaluated an antibiotic susceptibility approach using a variety of 14 d quadruple therapies, and demonstrated high success rates of 80%-100%. However, the results were not presented separately for first-line and rescue treatments [26].

Salvage (third-line) therapy

The published data for salvage therapy after failure of second-line treatment is very limited with 4 studies reporting on patient numbers in single figures[11,17-19]. Details are provided in the Table but it is difficult to draw any meaningful conclusions from the results.

Evidence from non-penicillin combination regimes in unselected groups of patients: Meta-analyses of trials on the efficacy of non-penicillin regimes in treating *H.pylori* infection are an alternate source of useful information when making treatment decisions about penicillin allergic individuals. These trials generally included unselected group of individuals without considering penicillin allergy status.

The meta-analysis by Gisbert *et al*[28] demonstrated a success rate of 81% with 7 d triple therapy regimen of PPI-Clarithromycin -Nitroimidazole, similar to the success rate with the regimen containing amoxicillin instead of nitroimidazole.

Two meta-analyses of randomised controlled trials on first line therapy of *H.pylori* with quadruple therapy of PPI-Bismuth-Tetracycline-Metronidazole have shown success rates of 77%[29,30]. A longer duration (10-14 d) of quadruple therapy was more effective than the 7 d triple therapy of PPI-Clarithromycin-Amoxicillin[30].

SUMMARY AND RECOMMENDATIONS

The triple therapy regimen of PPI-Clarithromycin-Metronidazole is still frequently used as first line therapy for penicillin allergic subjects[25]. However, whilst it demonstrates an acceptable success rate of approximately 80% in unselected patients [28], it does not perform well in penicillin allergic patients[11,14,18,20,21,23,25]. The reasons for this discrepancy are unclear, but it is possible is that the studies of unselected patients may only have had small numbers of penicillin allergic individuals, or the study design may have excluded individuals with antibiotic allergy. Whilst there is a paucity of recent data for this specific regimen, the efficacy of Clarithromycin-based triple therapy has been shown to be significantly impaired in

the presence of Clarithromycin resistance, which is an increasingly encountered issue [29]. Whilst increasing the duration of PPI-Amoxicillin-Clarithromycin triple therapy has been shown to improve success rates, this has not been demonstrated convincingly for the PPI-Clarithromycin-Metronidazole regimen[31]. If available, Vonoprazan could be considered as a substitute for PPI in clarithromycin-based triple therapy to improve its efficacy[20,21]. Sitafloxacin-based triple therapy is an alternative option, although this antibiotic is not widely available[17,19,20]. Bismuth-based quadruple therapy, lasting 10-14 d, is the most attractive option for first-line treatment of *H.pylori*, with a high success rate in patients with penicillin allergy[18,23,24,25], matching that in unselected patients[29,30]. In order to optimise the success of first line treatment, a detailed history of prior antibiotic use could aid the choice of regimen prescribed.

In the event of treatment failure, the published evidence suggests that second-line therapy should be instituted with the 10 d PPI-Levofloxacin-Clarithromycin regimen [14,18]; a Sitafloxacin-based triple therapy is an alternative option[17,19,20,27] . If Bismuth-based quadruple therapy has not been used as first-line treatment, then this regime could be considered for subsequent treatment, although there is variable evidence for the efficacy of PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[16,18,25]. Alternative antibiotic combinations may be more successful such as PPI-Bismuth-Tetracycline-Furazolidine[16] or PPI-Bismuth-Rifabutin-Ciprofloxacin [15], although there are concerns about the potential for side-effects with rifabutin, especially myelotoxicity[32].

It is not possible to provide any evidence-based recommendations for salvage therapy after failure of two treatment courses. It is generally recommended that in this situation, further treatment should be guided by the results of *H.pylori* culture and sensitivity testing[5,6,26]. Another approach is to confirm penicillin allergy at this stage, as many patients with this label turn out not to be truly allergic[5,6,9]. A negative penicillin skin test allows the safe use of amoxicillin-containing salvage regimens, as recommended for non-allergic patients.

CONCLUSION

This review of the evidence for treating *H.pylori* in penicillin-allergic individuals has highlighted the lack of high-quality studies to help guide management strategies. Whilst recommendations have been made based on the limited data, it would be important to monitor the success of treatment regimens and use what can be demonstrated to be effective locally[33]. Regional differences in drug availability will influence the choice of regimen, and patterns of antibiotic resistance rates will influence treatment success.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7669-7686

DOI: 10.3748/wjg.v27.i44.7669

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

ORIGINAL ARTICLE

Basic Study

Calycosin attenuates severe acute pancreatitis-associated acute lung injury by curtailing high mobility group box 1 - induced inflammation

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Institutional animal care and use committee statement: All animal experiments were conducted in accordance with relevant

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Abstract

BACKGROUND

Acute lung injury (ALI) is a common and life-threatening complication of severe acute pancreatitis (SAP). There are currently limited effective treatment options for SAP and associated ALI. Calycosin (Cal), a bioactive constituent extracted from the medicinal herb Radix Astragali exhibits potent anti-inflammatory properties, but its effect on SAP and associated ALI has yet to be determined.

AIM

To identify the roles of Cal in SAP-ALI and the underlying mechanism.

METHODS

SAP was induced *via* two intraperitoneal injections of L-arg (4 g/kg) and Cal (25 or 50 mg/kg) were injected 1 h prior to the first L-arg challenge. Mice were sacrificed 72 h after the induction of SAP and associated ALI was examined histologically and biochemically. An *in vitro* model of lipopolysaccharide (LPS)-induced ALI was established using A549 cells. Immunofluorescence analysis and western blot were evaluated in cells. Molecular docking analyses were conducted to examine the interaction of Cal with HMGB1.

guidelines and regulations and approved by the Animal Ethics Committee of The National Drug Clinical Trial Institution of The First Affiliated Hospital of Zhengzhou University (Ethic Review Number: 2019-KY-140).

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

Data sharing statement: No additional data are available.

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

Supported by the Key Research Projects of Henan Higher Education Institutions, No. 20A320064.

Country/Territory of origin: China

Specialty type: Immunology

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

Peer-review report's scientific quality classification

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

Received: May 17, 2021
Peer-review started: May 17, 2021

RESULTS

Cal treatment substantially reduced the serum amylase levels and alleviated histopathological injury associated with SAP and ALI. Neutrophil infiltration and lung tissue levels of neutrophil mediator myeloperoxidase were reduced in line with protective effects of Cal against ALI in SAP. Cal treatment also attenuated the serum levels and mRNA expression of pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6, IL-1β, HMGB1 and chemokine (CXC motif) ligand 1 in lung tissue. Immunofluorescence and western blot analyses showed that Cal treatment markedly suppressed the expression of HMGB1 and phosphorylated nuclear factor-kappa B (NF-κB) p65 in lung tissues and an *in vitro* model of LPS-induced ALI in A549 cells suggesting a role for HGMB1 in the pathogenesis of ALI. Furthermore, molecular docking analysis provided evidence for the direct interaction of Cal with HGMB1.

CONCLUSION

Cal protects mice against L-arg-induced SAP and associated ALI by attenuating local and systemic neutrophil infiltration and inflammatory response *via* inhibition of HGMB1 and the NF-κB signaling pathway.

Key Words: Severe acute pancreatitis; Acute lung injury; Calycosin; Mouse model; High-mobility group box 1; Nuclear factor-kappa B

Core Tip: In this study, we showed that Calycosin protects mice against L-arginine-induced severe acute pancreatitis (SAP) and associated acute lung injury (ALI) by attenuating local and systemic inflammatory response *via* inhibition of high mobility group box 1 (HGMB1) and the nuclear factor-kappa B signaling pathway. Suppression of HMGB1 expression is a potential target for the treatment of SAP-ALI.

Citation: Zhu CJ, Yang WG, Li DJ, Song YD, Chen SY, Wang QF, Liu YN, Zhang Y, Cheng B, Wu ZW, Cui ZC. Calycosin attenuates severe acute pancreatitis-associated acute lung injury by curtailing high mobility group box 1 - induced inflammation. *World J Gastroenterol* 2021; 27(44): 7669-7686
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7669.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7669

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease with wide clinical variation, resulting in an approximately 35% mortality when progressing to severe AP (SAP)[1]. Acute lung injury (ALI) is the most common cause of death in patients with severe AP (SAP), occurring in 10%-25% of SAP cases and responsible for up to 60% of AP-associated deaths[2]. Inflammation and pro-inflammatory cytokines play a key role in the development of SAP; therefore, inhibition of inflammation and the release of inflammatory factors are thought to be potential approaches for the therapy of SAP-ALI.

High mobility group box 1 (HMGB1), a highly conserved DNA binding nuclear protein, plays a vital role in the pathogenesis of inflammatory diseases such as pancreatitis[3]. Secreted HMGB1 released from necrotic acinar cells has been shown to aggravate the pancreatic inflammatory process[3,4]. Secreted HMGB1 exhibits cytokine-like properties that induces both the local and systemic inflammatory cascade that ultimately leads to multi-organ dysfunction[5,6]. HGMB1 has been shown to activate pro-inflammatory nuclear factor κB (NF-κB) signaling *via* interaction with multiple cell-surface receptors including Toll-like receptor (TLR) 2, TLR4 or TLR9 and receptor for advanced glycation end products (RAGE)[7]. The activation of NF-κB upregulates the gene expression of pro-inflammatory cytokines, chemokines and adhesion molecules which further aggravates the inflammatory response[4]. HMGB1 has also been shown to serve as a chemo-attractant recruiting neutrophils to site of

First decision: June 26, 2021
Revised: July 9, 2021
Accepted: September 10, 2021
Article in press: September 10, 2021
Published online: November 28, 2021

P-Reviewer: Jin CH, Lee SH, Samadder S, Şurlin VM
S-Editor: Wang LL
L-Editor: Webster JR
P-Editor: Wang LYT

inflammation and prevents neutrophil apoptosis which exacerbates tissue damage[8-10]. Blockade of HMGB1 by administration of anti-HMGB1 neutralizing antibodies was shown to inhibit the recruitment and accumulation of neutrophils in the lung[11, 12]. Thus, HMGB1 is a potential target for the treatment of ALI that is commonly found in SAP.

Calycosin (Cal) is one of the bioactive constituents extracted from the Chinese medicinal herb *Radix Astragali*, one of the five herbs of the Wutou Decoction, a classic herbal formula concocted by ancient Chinese medical doctor, Zhongjing Zhang, widely used for the treatment of rheumatoid arthritis[13]. Cal is a phytoestrogen isoflavone that has been shown to exhibit various biological effects including potent anti-inflammatory properties[14], as well as anti-cancer[15], neuroprotective[16], anti-Parkinson activity[17].

However, no studies have assessed the potential use of Cal for the expression of HMGB1 in the treatment of ALI in SAP. Hence, this study aims to address this question by exploring the effects of Cal administration on the expression of HMGB1 both in LPS induced ALI *in vitro* and an L-arginine induced ALI model in mice with SAP.

MATERIALS AND METHODS

Chemicals and reagents

L-arginine (L-arg: purity > 98%, endotoxin-free) and the BCA Protein Assay Kit were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Calycosin (Cal: $C_{16}H_{12}O_5$, purity > 98%;) was from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6, HMGB1, IL-1β and MPO were obtained from Wuhan Cloud-Clone Corp. (Wuhan, China). ELISA kits for tumor necrosis factor (TNF)-α and CXCX-1 were procured from Proteintech Group (Rosemont, IL, United States). The amylase ELISA kit was bought from Shanghai BlueGene Biotech Co., Ltd. (Shanghai, China). Primary antibodies against NF-κB p65 (p65), phosphorylated NF-κB p65 (p-p65), and GAPDH were purchased from Cell Signaling Technology Inc. (Danvers, MA, United States). Primary antibody against lymphocyte antigen 6 complex locus G6D (Ly6G) was obtained from Abcam (Cambridge, United Kingdom). Primary antibody against HMGB1 and Fluorescent secondary antibody were produced by Proteintech Group. Horseradish peroxidase (HRP)-conjugated secondary antibodies and Hypersensitive WB Chemiluminescent Substrate Reagent were from Beyotime Biotechnology (Jiangsu, China).

Animals

Twenty-four male C57BL/6N mice (weight: 18-22 g, age: 8–10 wk) were purchased from Charles River Company (Beijing, China). The mice were housed in a specific pathogen-free facility with a dark/light cycle of 12/12 h in ambient temperature of 22 ± 2 ℃ and humidity of 50% ± 10%. Mice were fed standard rodent chow and clean water *ad libitum*. All animal experiments were conducted in accordance with relevant guidelines and regulations and approved by the Animal Ethics Committee of The National Drug Clinical Trial Institution of The First Affiliated Hospital of Zhengzhou University (Ethic Review Number: 2019-KY-140). All mice received humane care and the study was conducted following the ARRIVE guidelines.

Murine model of L-arginine-induced severe acute pancreatitis

L-arg was dissolved in normal saline and then sterilized by filtration (pH approximately equal to 7.0). Mice were randomly divided into four groups (n = 6 in each group): Control (Saline), L-arg (4 g/kg + Saline), L-arg + Low-dose Cal (L, 25 mg/kg bodyweight), and L-arg + High-dose Cal (H, 50 mg/kg bodyweight). The Cal treatment groups received prophylactic Cal treatment (25 or 50 mg/kg) *via* intraperitoneal injection 1 h before the first injection of L-arg. The Control and L-arg groups received an intraperitoneal injection of normal saline before L-arg injection. After the 1 h prophylactic treatment, the mice received intraperitoneal injections of either normal saline (Control group) or 4 g/kg of L-arg every hour for 2 h to induce severe AP as previously described by Dawra *et al*[18]. Blood samples were drawn from the retro-orbital venous plexus under general anesthesia using sodium pentobarbital. Blood samples were centrifuged at 3000 rpm for 15 min at 4°C and plasma serum stored at -80°C for downstream biochemical analyses of serum amylase and cytokine levels. Mice were then sacrificed, and the pancreas and lung tissues from each mouse were

quickly removed. The pancreas and left lung were dissected in two with one half being fixed in 4% paraformaldehyde (PFA) for histopathological assessment and the other half snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Measurement of wet-to-dry weight ratio in lung tissue

Lung tissue wet-to-dry weight (W/D) ratio was employed to determine the extent of pulmonary edema following L-arg administration. The right lung was excised and surface water was removed by blotting with filter paper. The lung weight was immediately measured on a standard electronic laboratory scale and recorded as the wet weight (W). The lung was then dried in an oven at 60°C for 48-72 h and reweighed as dry weight. The W/D ratio was calculated based on the following formula: W/D = (wet weight − dry weight)/dry weight.

Histopathological assessments

Fixed pancreatic and lung tissues were embedded in paraffin blocks and 4 μm thin sequential sections were prepared. Tissue sections were stained with hematoxylin and eosin as per our standard laboratory protocol. Stained sections were visualized and imaged under an optical light microscope (CX31, Olympus Optical Co., Ltd., Japan), and histopathological changes were assessed by three experienced pathologists who were blind to the experimental procedure. Pancreatic tissue damage was graded using a modified Schmidt Scoring System[19] as normal to severe (scale of 0-4) based on the degree of inflammatory cell infiltration, vacuolization and acinar cell necrosis. Similarly, lung tissue sections were assessed for alveolar thickening and inflammatory cell infiltration with scoring system ranging from 0-3[20].

Immunohistochemical evaluation of Ly6G expression

For immunohistochemical staining, lung tissue sections were deparaffinized and rehydrated in graded ethanol. Sections were immersed in 3% hydrogen peroxide (in methanol) for 20 min to block endogenous peroxidase activity, and then boiled in 0.1% citrate buffer for antigen retrieval, followed by incubation in 3% BSA serum (in PBS) for 30 min at room temperature to block non-specific immuno-reactivity. Tissue sections were incubated overnight at 4°C with anti-Ly6G antibody (1:500 dilution in 3% BSA-PBS) to stain neutrophils. Following incubation with HRP-conjugated secondary antibody color development was achieved by incubating sections with diaminobenzidine color development reagent and visualized under an optical light microscope. Five non-overlapping high-power fields (×100 magnification) for each section were captured. The integrated optical density (IOD) of positive expression for Ly6G in lung tissue sections were measured from gray-scale images using Image J software (Image J 1.52, National Institute of Health) following calibration of hue (0 – 25), saturation (0-255), and intensity (0-255) levels in the area of interest. The AOD (relative expression) was determined as IOD/positive area.

Serum enzymes and cytokine quantification using ELISA

Blood samples collected were subjected to ELISA analysis to determine serum levels of amylase, TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 in accordance with the corresponding manufacturer's protocol. Serum amylase level was expressed as U/L and serum TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 Levels were expressed as pg/mL.

MPO activity in lung tissues

To assess lung MPO activity, 20 mg of frozen lung tissue were homogenized in homogenization buffer (0.5% hexadecyl trimethylammonium bromide, 5 mmol/L EDTA, and 50 mmol/L potassium phosphate buffer; pH 6.2) on ice. The homogenate was then centrifuged at 12000× g for 15 min at 4°C and the resulting supernatant was retained. MPO activity was determined in accordance with the corresponding manufacturer's protocol, MPO levels were expressed as pg/mg.

In vitro model of LPS-induced ALI in A549 cells

The lung adenocarcinoma A549 cell line was purchased from ATCC (VA, United States) and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 100 IU/mL streptomycin and 100 IU/mL streptomycin. An *in vitro* cellular model of ALI was established by treating A549 cells with 1 μg/mL lipopolysaccharide (LPS, Sigma, United States) for 24 h[21]. To test the effects of Cal, cells were pretreated with various concentrations of Cal (1 μM, 5 μM, 10 μM, and 20 μM) for 1 h before LPS stimulation. After 24 h of stimulation, cells were either fixed for immunofluorescence assessment, or harvested for protein extraction for downstream western blot analyses.

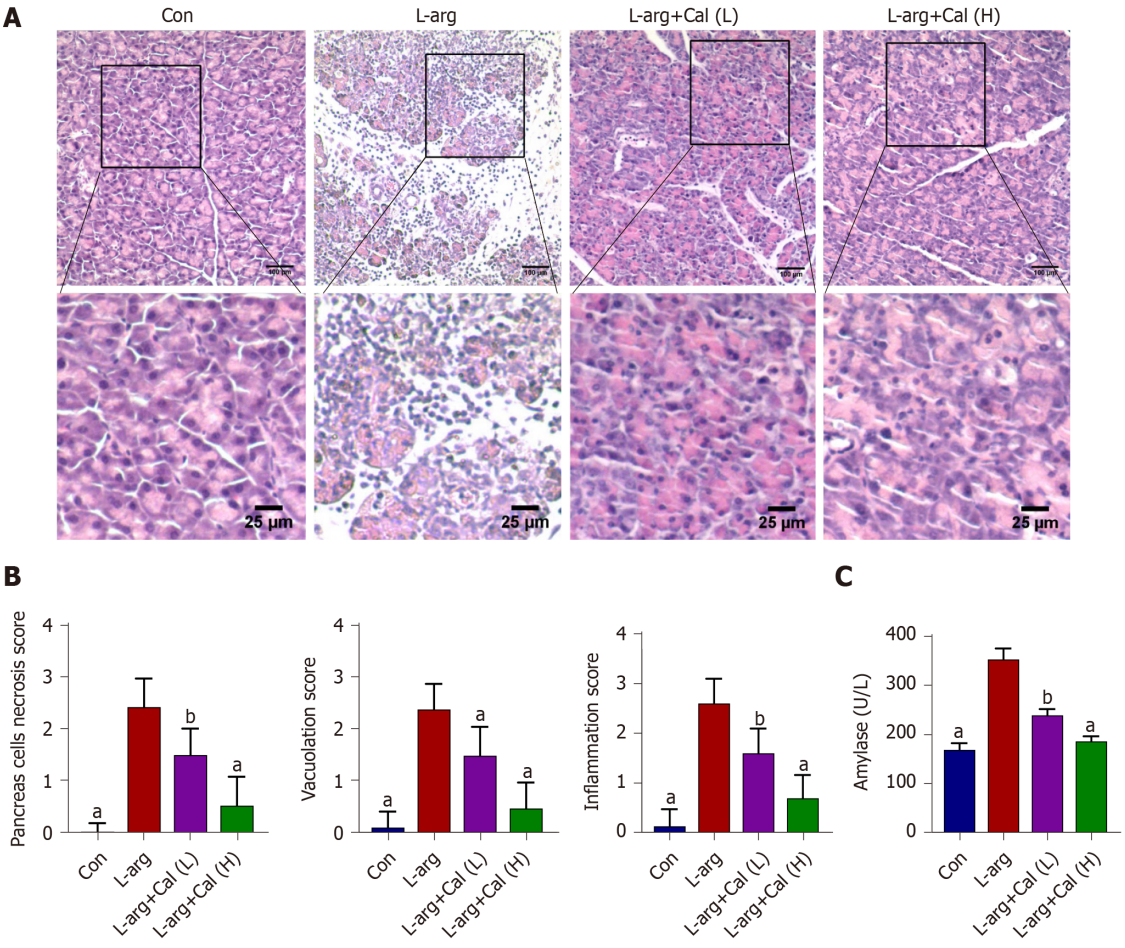

Figure 1 Effects of calycosin on L-arginine induced severe acute pancreatitis. Mice were pretreated with calycosin [calycosin (Cal): 25 and 50 mg/kg] for 1 h, L-arginine (L-arg: 4 g/kg) was intraperitoneally (*i.p.*) injected every hour for two consecutive hours. A: The histological assessment of pancreatic tissue damage in the control (Con), L-arg and Cal group (L: 25 mg/kg; H: 50 mg/kg); B: Pancreatic histological scores; C: Serum amylase and lipase levels. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

CCK-8

The CCK-8 assay was used to assess the effects of Cal on A549 cell viability. Cells were seeded onto 96-well plates at a density of 1×10^{4} cells/well and then treated with various concentrations of Cal (1, 5, 10 and 20 μM) for 24 h. DMSO (0.1%) was added to the 0 μM group. After treatment, the cells were incubated with 10 μl of CCK-8 reagent for 1 h (Bosterbio, United States) and then the absorbance at 450 nm was measured using a Microplate Reader.

RT-qPCR

Total RNA was extracted using TRIzol reagent. Two microgram of total RNA was reversed transcribed into cDNA using Hiscript II Q RT SuperMix for qPCR in accordance with the manufacturer's instructions (Nanjing, China). Real-time quantitative PCR was carried out using the SYBR Green qPCR Master Mix, containing template cDNA and specific primers for TNF-α, IL-6, IL-1β, CXCL-1, or HMGB1. PCR reactions were carried out on an ABI Prism Real-time PCR System (Applied Biosystems, Foster City, CA, United States). GAPDH was used as an internal housekeeping control. The primers used are listed in Supplementary Table 1.

Immunofluorescence staining

The expression of HMGB1 and NF-κB (p-p65) in A549 cells and lung tissue was evaluated by immunofluorescence. Briefly, cells was washed twice with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min, and then permeabilized with 0.5% Triton X-100 in TBS-Tween 20 (TBST) for 5 min. For lung tissues, sections were dewaxed, hydrated, and treated with EDTA-containing antigen retrieval buffer (pH 8.0) in a microwave oven, and then blocked with 5% BSA for 1 h. Samples (cells or tissue sections) were then incubated with the primary antibodies (HMGB1, 1:100; or NF-κB

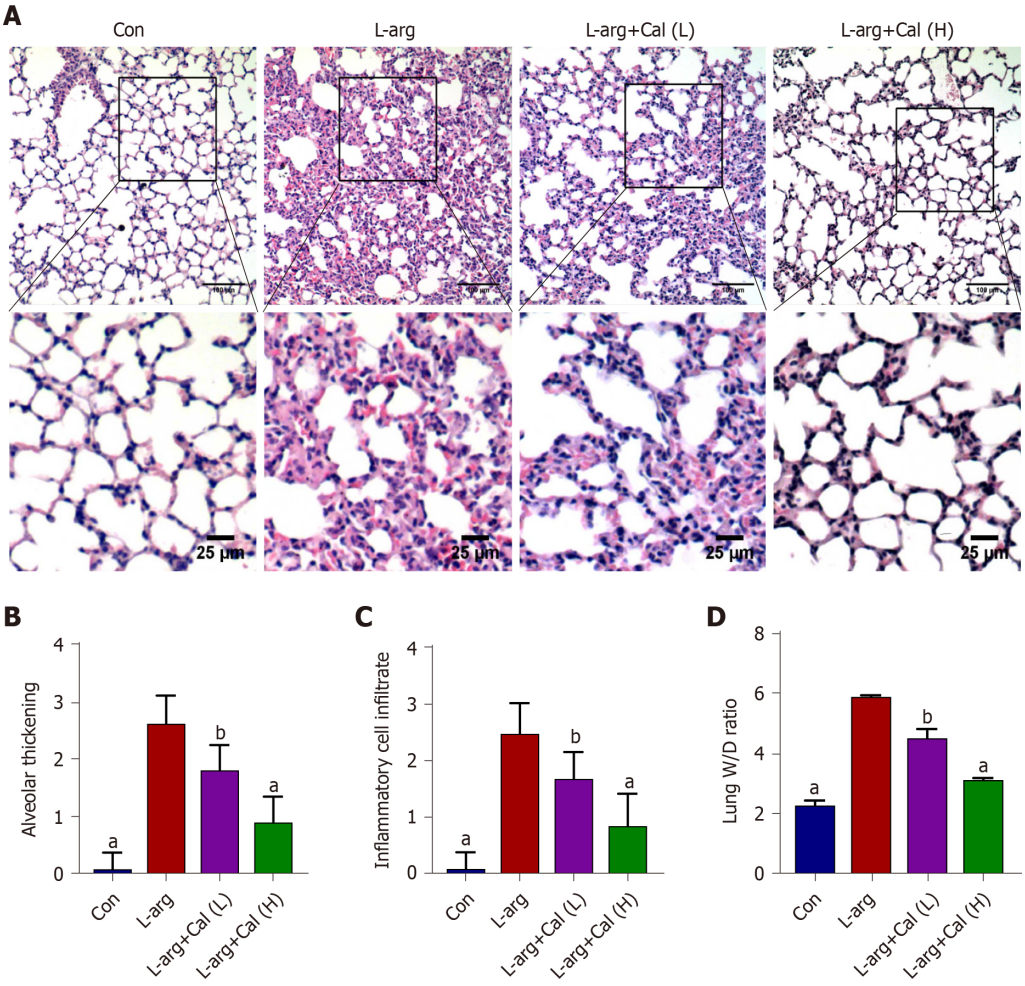

Figure 2 The effects of calycosin on histopathological findings of lung tissue in mice. H&E sections were examined by light microscopy. A: Representative pathological images of the lung tissue; B: Lung injury scores in alveolar thickness; C: Inflammatory infiltrate; D: The lung wet/dry weight ratios are shown. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

p-p65, 1:200) in TBST overnight at 4°C. Samples were washed three times with TBST and then incubated with Cy3–conjugated Affinipure Goat Anti-Rabbit IgG (H + L) antibody (1:100, Proteintech) for 1 h. After washing three times with TBST, the samples were incubated with DAPI to stain the nuclei and then mounted in anti-fade mounting medium for assessment by fluorescence microscopy.

Western blot analysis

Frozen cells or lung tissues were lysed and homogenized in RIPA lysis buffer containing protease inhibitors and 1 mmol/L phenylmethanesulfonylfluoride (PMSF) on ice. Lysates were then mixed with SDS loading buffer and denatured by heating at 100°C for 10 min. Each protein sample was separated by SDS-PAGE electrophoresis (10% gel), and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The following primary antibodies and dilutions were used: anti-p65 (1:1000), anti-p-p65 (1:1000), anti-HMGB1 (1:500) and anti-GAPDH (1:1000). Following extensive washing with TBST the membranes were incubated with the HRP-conjugated secondary antibody (1:1000 in 1% (w/v) skim milk in TBST) for 1 h at room temperature. Protein-antibody immunoreactivity was detected by Hypersensitive Chemiluminescent Reagent and imaged on a LI-COR Odyssey Imaging System (LI-COR, Lincoln, NE, United States). Densitometry analysis was performed using the associated software and the band intensity of target proteins was normalized to GAPDH signals.

Molecular docking

Molecular docking analysis of the interaction(s) between Cal and HMGB1 was carried out using the open-source Autodock Vina v1.1.2 software (Scripps Research, CA,

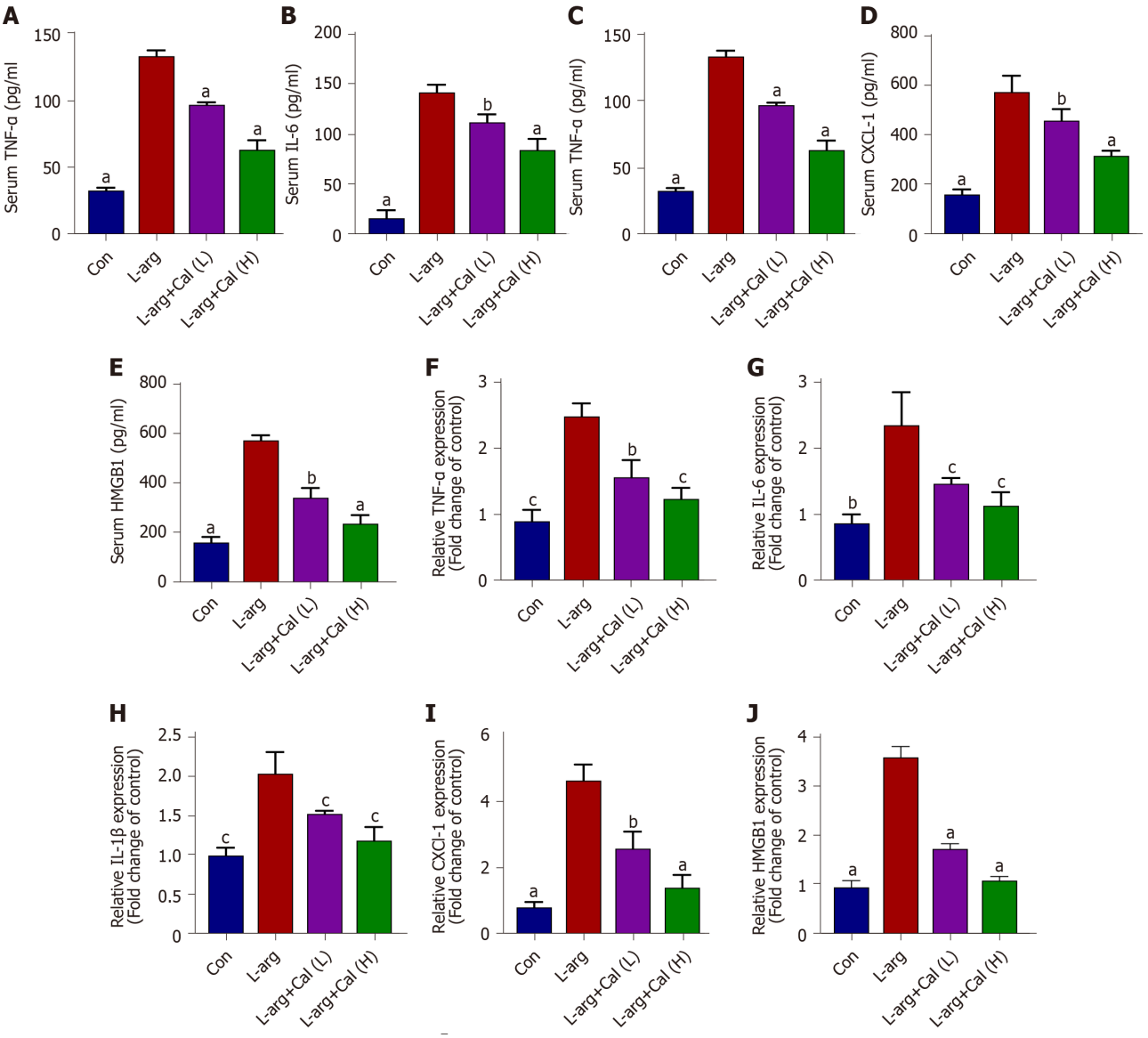

Figure 3 Effect of calycosin on tumor necrosis factor α, interleukin-6, interleukin-1β, chemokine ligand 1 and high mobility group box 1 in serum and mRNA levels. A: TNF-α; B: IL-6; C: IL-1β; D: CXCL-1; E: HMGB1 in serum levels were determined by ELISA kits. F: TNF-α; G: IL-6; H: IL-1β; I: CXCL-1; J: HMGB1 in mRNA levels. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$ *vs* L-arg group. TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6; IL-1β: Interleukin-1β; HMGB1: High mobility group box 1; CXCL-1: Chemokine ligand 1; L-arg: L-arginine; Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

United States). Two-dimensional (2D) coordinates of Cal were retrieved through the PubChem website (https://pubchem.ncbi.nlm.nih.gov). The three-dimensional structure of HMGB1 A-box (PDB ID: 2RTU) was retrieved from the RCSB Protein Data Bank. Optimized binding conformations were generated using criteria such as energy minimization and cluster size. To increase the accuracy of the binding conformations generated, the value of exhaustiveness was set to 1. Finally, the superposition of Cal and HMGB1 was carried out. Receptor proteins and ligand molecules were converted into PDBQT formats.

Statistical analysis

All bar graphs presented in this study are expressed as mean ± SD values of at least three independent experiments. Differences between 4 groups were compared by one-way analysis of variance using GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, United States). Statistical significance was set at a P value < 0.05 unless otherwise stated.

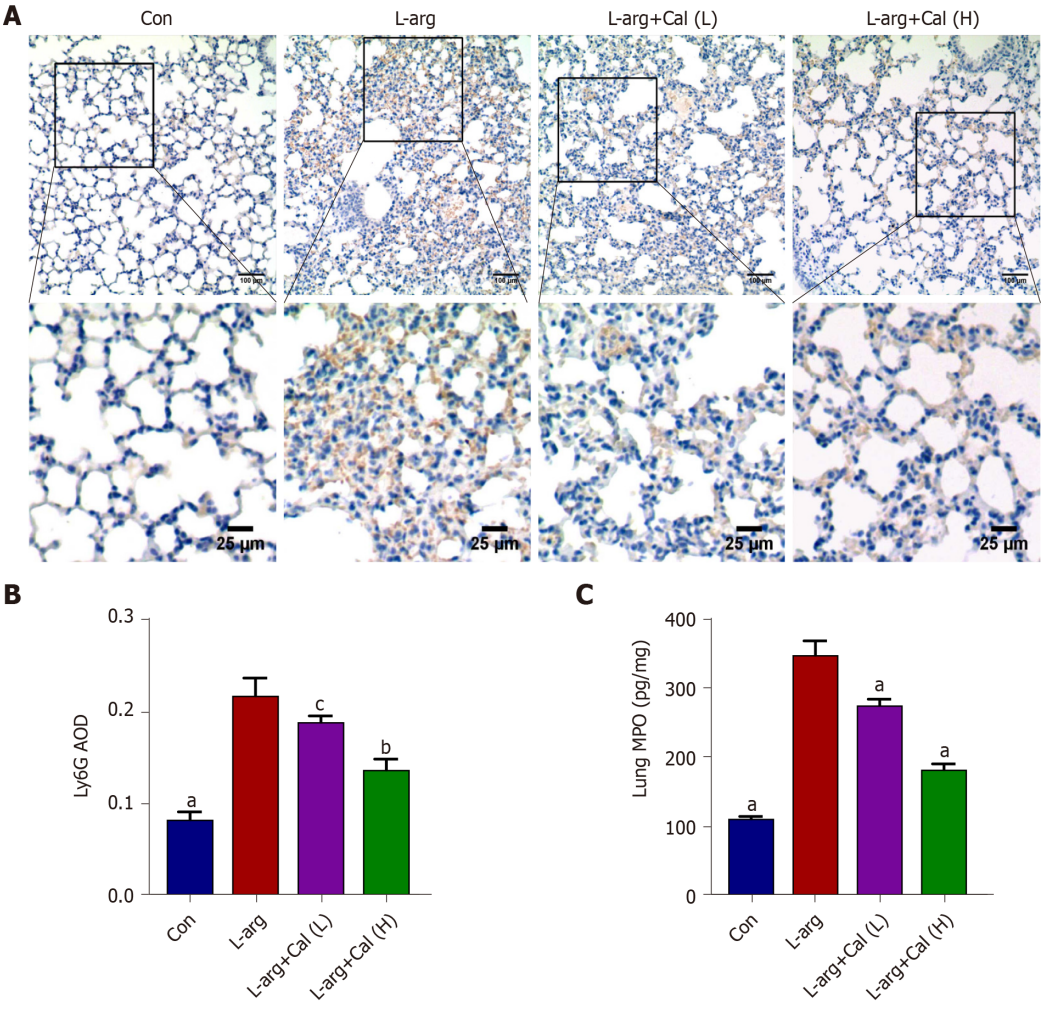

Figure 4 The effect of calycosin on the immunological staining of lung slices for Ly6G. A: Micrographs of lung section stained with immunological staining of lung slices for Ly6G (brown); B: Average optical density for Ly6G was determined; C: Levels of myeloperoxidase in per milligram lung tissue were determined by enzyme-linked immunosorbent assay kits. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

RESULTS

Cal treatment protects against ALI in mice with SAP

As shown in Figure 1A and B, the pancreas from mice treated with L-arg exhibited classical histological signs of SAP including significant tissue damage, acinar cell vacuolization and necrosis, as well as abundant inflammatory cell infiltration when compared with control mice ($P < 0.001$). On the other hand, mice that were administered prophylactic Cal throughout the experimental period showed a dose-dependent reduction in pancreatic tissue damage (Figure 1A). Elevated serum amylase is a key indicator of pancreatic acinar cell injury in AP. Amylase activity was markedly elevated (more than 2-fold) at 72 h following the induction of SAP in the L-arg group when compared to the control group (Figure 1C; $P < 0.001$). Consistent with histological findings, Cal administration dose-dependently decreased serum amylase levels when compared to the L-arg SAP group ($P < 0.001$). Taken together, these results indicated that Cal administration can protect mice against L-arg-induced SAP.

SAP is often accompanied by ALI and contributes to the majority of AP-associated death[22,23]. We examined whether secondary ALI was similarly induced in our SAP mouse model following L-arg treatment. As shown in Figure 2A-C, lung tissue from control mice showed normal pulmonary architecture, while the lung tissue in the L-arg SAP group exhibited significant lung edema, alveolar wall thickening, local hemorrhage, and inflammatory cell infiltration ($P < 0.001$). In contrast, the aforementioned histopathological observations in the Cal treatment groups exhibited noticeable improvement particularly when mice were treated with 50 mg/kg Cal (high-dose). Alveolar capillary permeability or pulmonary edema was assessed using the W/D

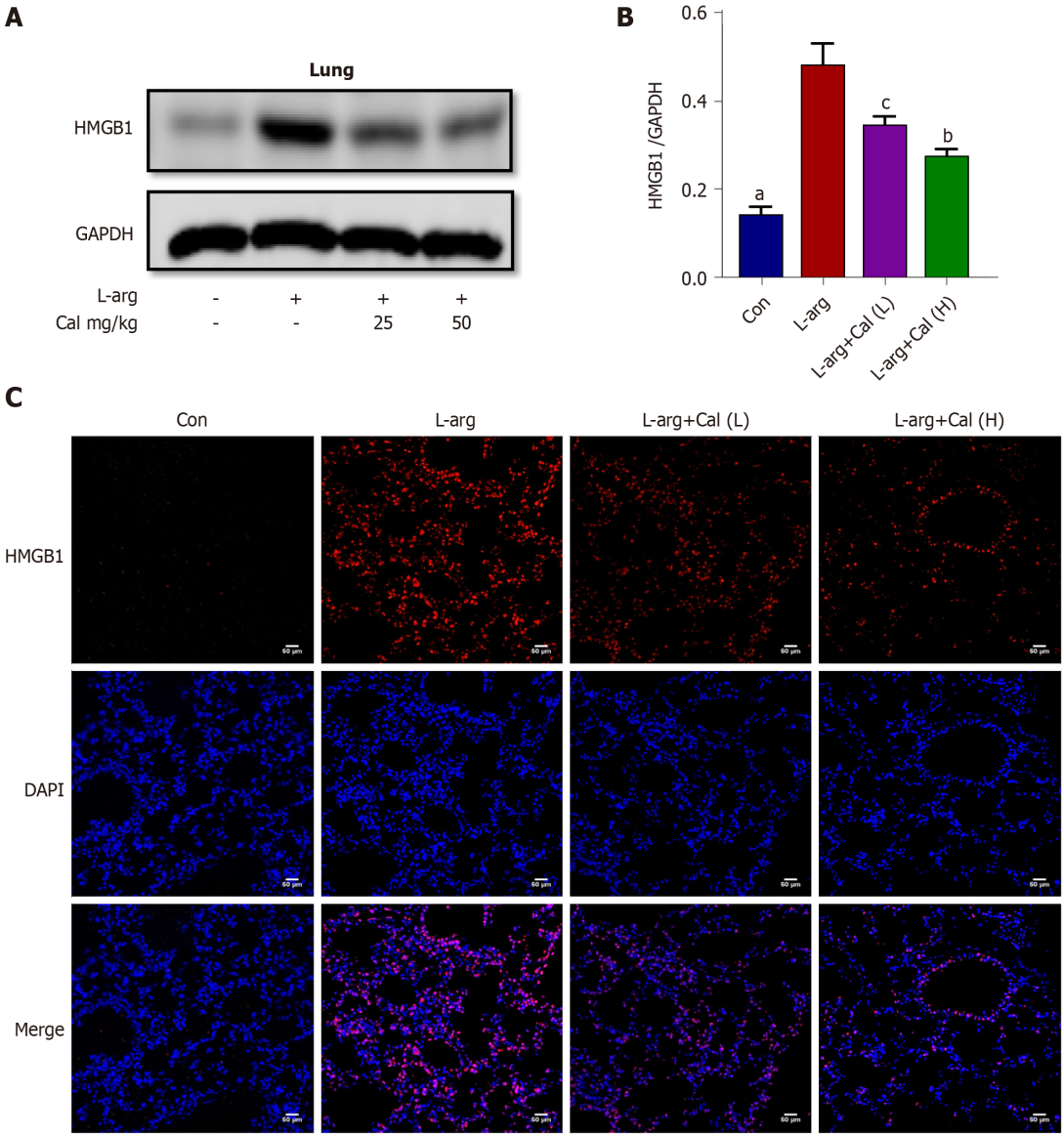

Figure 5 Effects of calycosin on the expression of high mobility group box 1 in lung tissues. A: Western blot detection of high mobility group box 1 (HMGB1) expression in lung tissue; B: Quantitative analysis of HGMB1 in lung tissue; C: Immunofluorescence staining of HMGB1 in lung tissue. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$, *vs* L-arg group. L-arg: L-arginine; HMGB1: High mobility group box 1; Con: Control group; Cal: Calycosin group.

ratio. Consistent with secondary ALI, L-arg SAP mice exhibited a markedly elevated W/D ratio compared with controls (Figure 2D; $P < 0.001$), whereas Cal treatment significantly reduced pulmonary edema (lower W/D ratio) in a dose-dependent manner. Together, our results showed that Cal treatment can further alleviate secondary ALI in mice with SAP.

Cal inhibits pro-inflammatory cytokine expression and secretion

The expression and release of pro-inflammatory cytokines and mediators are critical effectors that exacerbate local pancreatic tissue damage and mediate systematic inflammation during SAP[24]. Thus, serum levels of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 were determined using ELISA and their mRNA levels in lung tissue were also determined. Consistent with histological observations, serum levels of TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 were markedly elevated in the L-arg SAP group when compared with controls (Figure 3A-E, respectively; $P < 0.001$) as well as their mRNA expression levels (Figure 3F-J). In contrast, Cal treatment dose-dependently reduced the secretion and serum concentration of these pro-inflammatory cytokines and chemokine when compared with the L-arg SAP group.

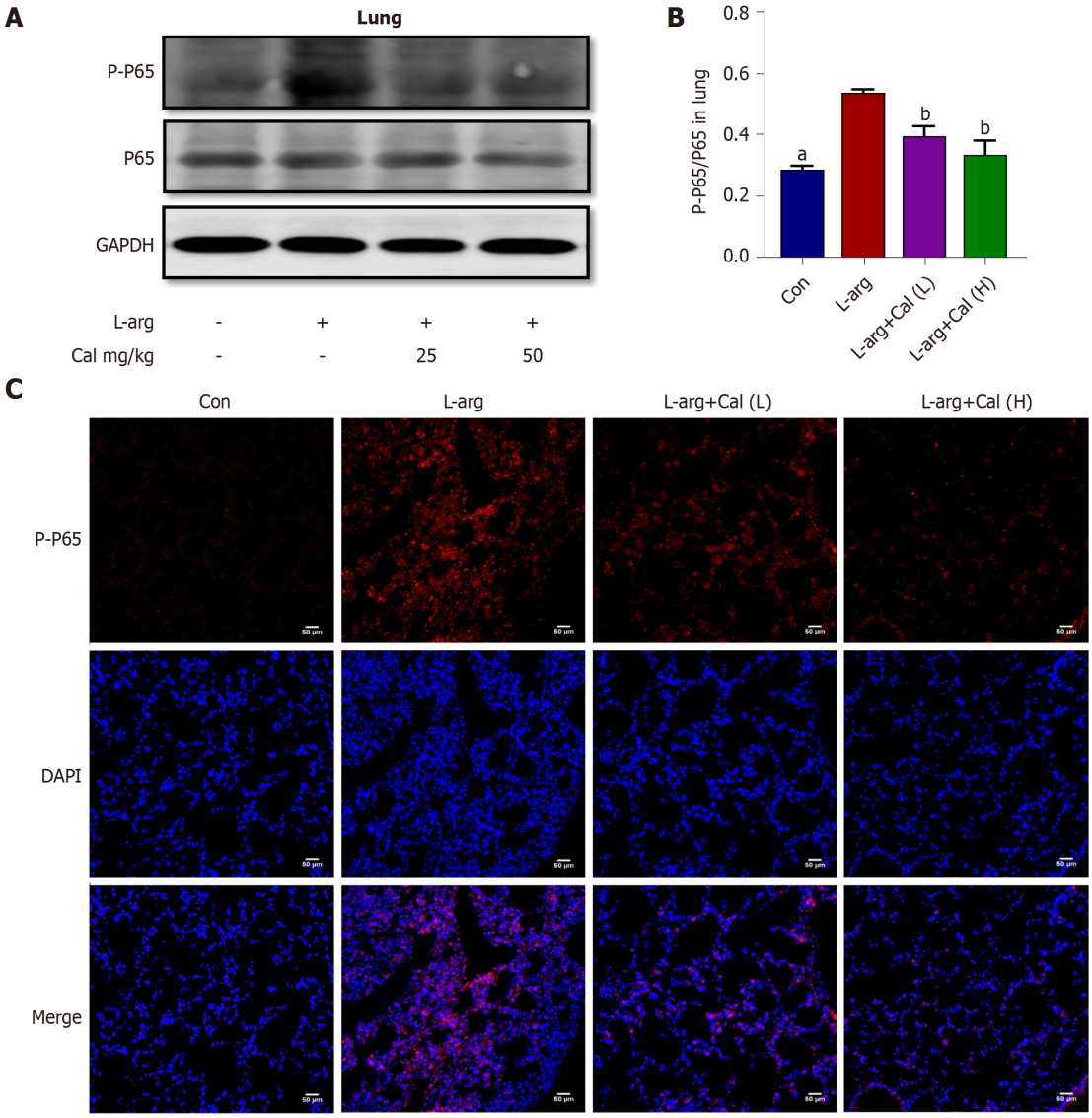

Figure 6 Effects of calycosin on the expression of phosphorylated nuclear factor-kappa B-p65 expression in lung tissues. A: Western blot detection of phosphorylated nuclear factor-kappa B-p65 (NF-κB-p65) expression in lung tissue; B: Quantitative analysis of phosphorylated NF-κB-p65 in lung tissue; C: Immunofluorescence staining of phosphorylated NF-κB-p65 in lung tissue. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. p-p65: Phosphorylated nuclear factor-kappa B-p65; Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

Cal prevented neutrophil infiltration in the lung

Previous studies have shown that the accumulation of a large number of infiltrating neutrophils in the lungs is a pathophysiological feature of ALI[7,8]. We assessed whether this corresponded with reduced neutrophil infiltration in the lungs. To this end, lung tissue sections were stained for Ly6G, a specific marker that separates neutrophils from other inflammatory cells such as leukocytes[25]. As shown in Figure 4A and B ($P < 0.001$), a significantly greater number of Ly6G-positive cells was observed in L-arg SAP mice than in control mice. This elevation in Ly6G positivity in the L-arg SAP group was also correlated with marked increases in lung MPO activity (Figure 4C; $P < 0.001$) further attesting to significant neutrophil infiltration in the lungs following L-arg-induced SAP. On the other hand, Cal treatment dose-dependently reduced neutrophil infiltration as demonstrated by reduced Ly6G positivity in lung tissues (Figure 4A and B; $P < 0.05$ and $P < 0.01$) and diminished lung MPO activity (Figure 4C; $P < 0.001$). These results further strengthened the protective effects of Cal against secondary ALI associated with AP.

Cal suppressed the activation of HMGB1 and the NF-κB signaling pathway in vivo

Previous studies have shown that many of the pro-inflammatory effects of extracellular HMGB1 is driven by activation of the NF-κB signaling pathway[3-4]. To examine whether NF-κB signaling is involved in mediating the inflammatory effects of

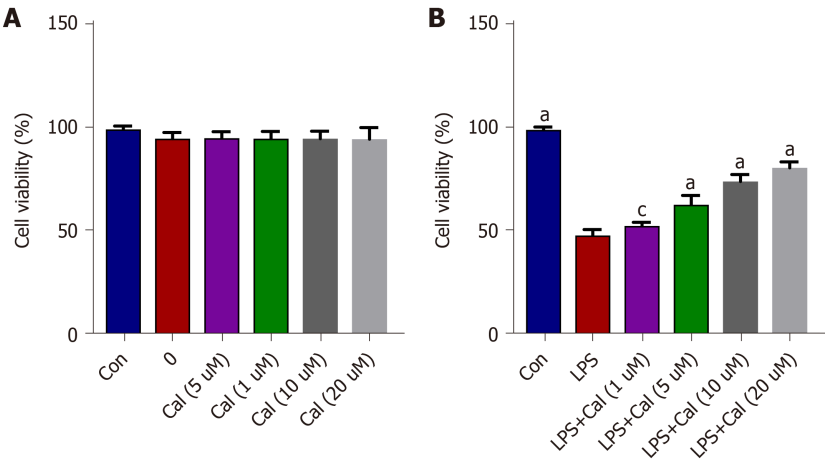

Figure 7 Effects of calycosin on the cell viability of A549 cells. A: Cell vitality was detected by the CCK8 assay after calycosin Cal (1 μM, 5 μM, 10 μM, and 20 μM) treatment; B: Cal pretreatment markedly increased cell vitality induced by lipopolysaccharide (LPS, 1 μg/mL). Data represent mean ± SD values. $^{a}P < 0.001$ *vs* LPS group; $^{c}P < 0.05$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

HMGB1 in ALI *in vivo*, we examined HMGB1 and NF-κB expression and signaling activation using immunofluorescence and western blot analyses, respectively. Our results showed that the expression of HMGB1 (Figure 5A-C) and p-p65 (activated form of NF-κB p65 subunit) (Figure 6A-C) was significantly elevated ($P < 0.001$) in lung tissues following L-arg induced SAP. Treatment with Cal resulted in a dose-dependent decrease in HMGB1 and p-p65 expression in the lungs. These results suggest that the inhibition of HMGB1, NF-κB signaling activation and pro-inflammatory cytokine secretion is in part associated with the protective effect of Cal against ALI in mice with SAP.

Cal treatment improved LPS-induced A549 cells viability and inflammatory response

To further define the molecular mechanism of the protective effects of Cal against ALI, an *in vitro* model of ALI was established by stimulating the lung adenocarcinoma cell-line A549 with LPS in the absence or presence of Cal. As shown in Figure 7A, treatment with the indicated concentrations of Cal for 24 h did not exert detrimental effects on A549 cell viability (Figure 7A). On the other hand, treatment with LPS (1 μg/mL) markedly reduced A549 cell viability as compared with untreated controls, while treatment with Cal resulted in a dose-dependent enhancement of cell viability especially at 20 μM (Figure 7B). Based on these data, 20 μM of Cal was used for downstream investigations.

Cal suppressed the activation of HMGB1 and the NF-κB signaling pathway in vitro

The effects of Cal on HMGB1 and NF-κB p65 expression and activation were examined using the LPS-induced ALI cellular model. Immunofluorescence and western blot analyses demonstrated that LPS stimulation markedly induced HMGB1 and p-p65 expression (Figure 8 and Figure 9; $P < 0.001$) and this was markedly attenuated following treatment with Cal.

The theoretical binding mode of the Cal and HMGB1 pathway

The interaction between HMGB1 A-box and Cal was assessed using molecular docking software. The HMGB1 structure consists of the A-box and B-box, two DNA binding domains and a negatively charged acidic C-terminal tail. The B-box fragment has a pro-inflammatory effect, while the A-box fragment is known to be antagonistic to inflammatory responses, when the A-box binds to the TLR4 receptor but is not able to trigger TLR4 dimer formation due to a lack of critical interactions with the TLR4[26]. As shown in Figure 10, amino acid residues Arg73, and Gly14 of HMGB1 interacts with Cal to form hydrogen bonds. Amino acid residues Glu77, Lys11, Lys10, Asp8, Arg8, Arg13, and Pro12 of the HMGB1 A-box form hydrophobic interactions with Cal. These molecular docking results suggest that Cal exhibits distinct binding affinities to the HMGB1 A-box that inhibited its downstream signaling pathways.

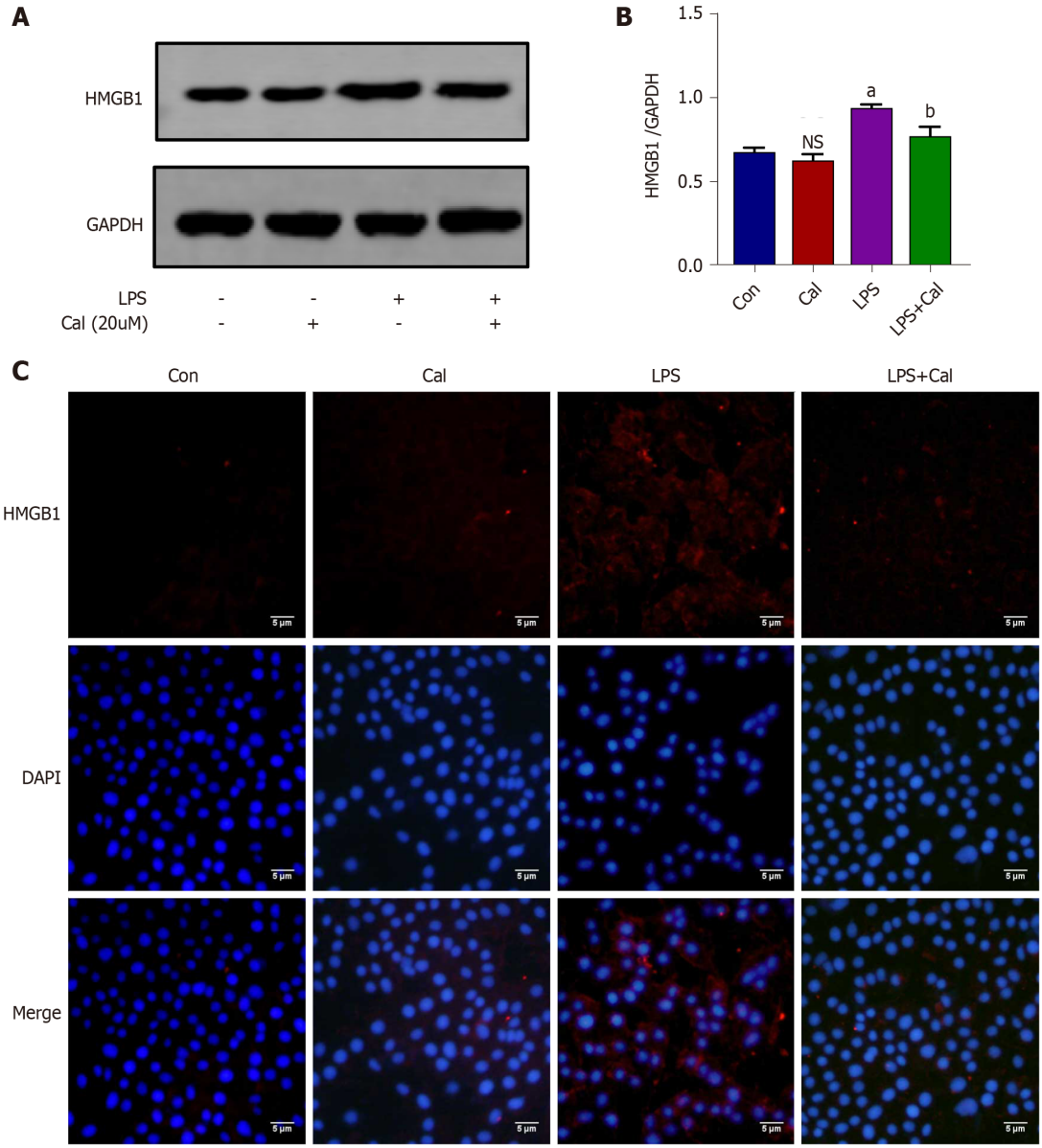

Figure 8 Effects of calycosin on the expression of high mobility group box 1 *in vitro* model of LPS-induced ALI using A549 cells. A: Western blot detection of high mobility group box 1 (HMGB1) expression in A549 cells; B: Quantitative analysis of HMGB1 in A549 cells; C: Immunofluorescence staining of HMGB1 in A549 cells. Control group (untreated), Calycosin group (Cal, 20 μM), LPS group and LPS + Cal group were treated with 1 μg/mL LPS for 24 h in the absence or presence of Cal (20 μM) pretreatment for 1 h. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* control group; $^{b}P < 0.01$ *vs* LPS group. ns: No significance *vs* Con group. HMGB1: High mobility group box 1; Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

DISCUSSION

SAP is one of the most common causes of acute abdominal pain and often manifests with many complications, resulting in high mortality[1]. The pathogenesis of AP is multifactorial and involves a multi-step process. In the early stages of the disease, intra-pancreatic activation of pancreatic enzymes such as trypsin, leads to auto-digestion of acinar cells and initiates the production and release of various pro-inflammatory mediators (or cytokine cascade). The elevation in pancreatic pro-inflammatory cytokine levels induces pancreatic oxidative stress and increases vascular permeability, leading to pancreatic edema and acinar cell necrosis, which augments pancreatic inflammation[27,28]. At this stage, inflammatory cell infiltration and macrophage activation results in the further release of systemic cytokines and inflammatory mediators, leading to systemic inflammatory response syndrome. ALI is a common and severe complication associated with SAP[29]. Thus, identifying effective therapies that can effectively treat local and systemic tissue damage remains an unmet medical challenge.

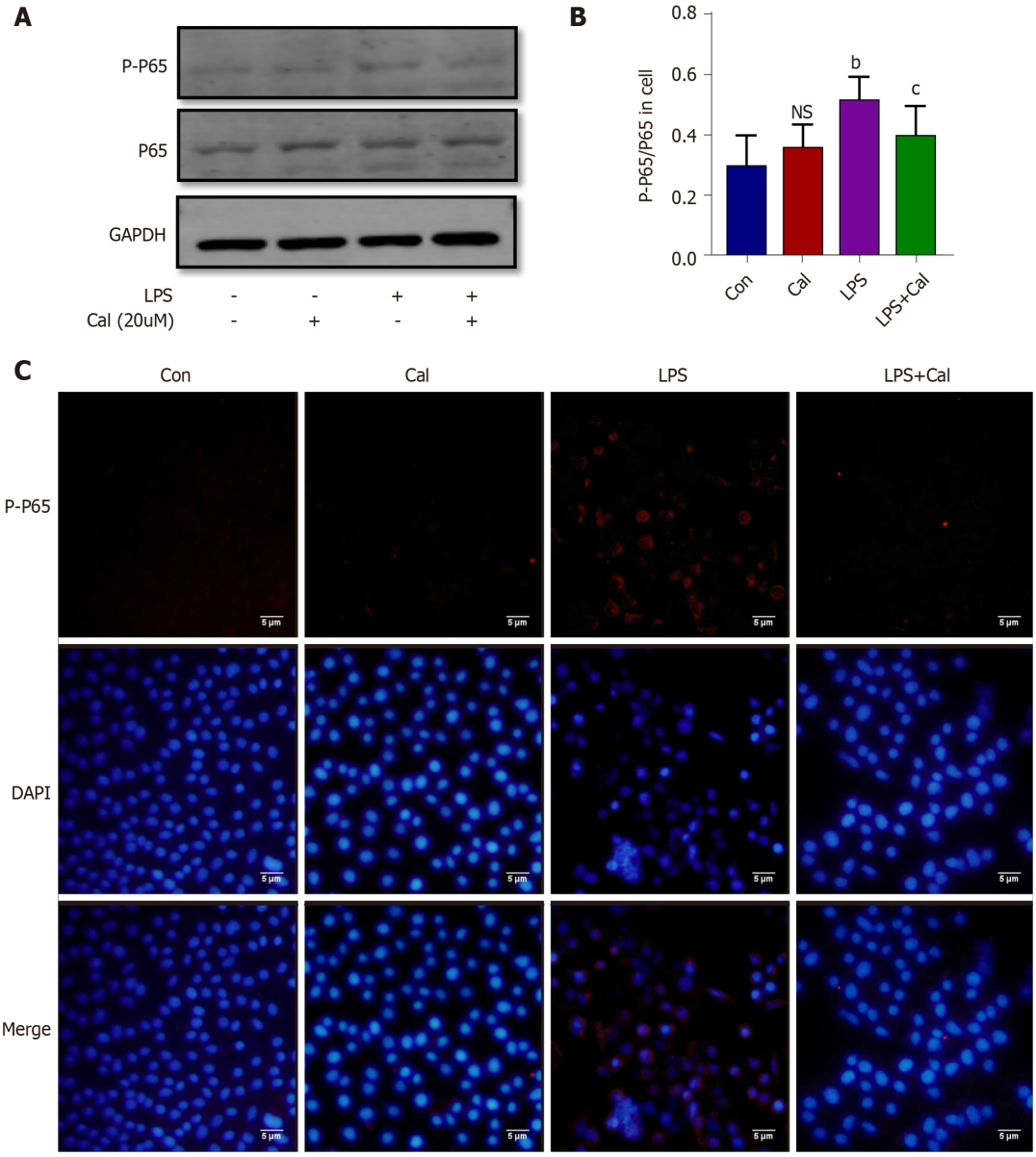

Figure 9 Effects of calycosin on the expression of phosphorylated nuclear factor-kappa B-p65 expression in the *in vitro* model of LPS-induced ALI using A549 cells. A: Western blot detection of phosphorylated nuclear factor-kappa B-p65 (NF-κB-p65) expression in A549 cells; B: Quantitative analysis of phosphorylated NF-κB-p65 in A549 cells; C: Immunofluorescence staining of phosphorylated NF-κB-p65 in A549 cells. Data represent mean ± SD values; $^{b}P < 0.01$ *vs* Con group; $^{c}P < 0.05$ *vs* LPS group. Ns: No significance *vs* Con group. p-p65: Phosphorylated nuclear factor-kappa B-p65; Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

ALI is the most common extra-pancreatic complication leading to death in SAP patients, and there is no consensus on the most effective treatment[3]. Therefore, we aimed to explore the effect of Cal on ALI in SAP. We showed that Cal extracted from the Chinese medicinal herb *Radix astragali* effectively protected mice against L-arg-induced ALI in SAP. L-arg-induced SAP is a well-established model of pancreatitis that reflects the pathological changes seen in humans[18]. We found that mice prophylactically treated with Cal exhibited a marked decrease in serum amylase and tissue MPO activity, pronounced reductions in pancreatic and pulmonary lung tissue damage, and significantly diminished pro-inflammatory cytokine production in part due to inhibition of the HMGB1/NF-κB signaling axis. The molecular docking analysis results suggest that Cal directly binds HMGB1 *via* hydrogen bonds and hydrophobic interactions *via* multiple residues on HMGB1. However, the implication of these residues on HMGB1 activity and function remains to be elucidated.

We first assessed the effect of Cal on the histopathological injury in pancreas and lung tissue. The results revealed severe pancreatic histoarchitectural changes, including pancreatic edema and vacuolization, acinar cell necrosis, and inflammatory cell infiltration. The incidence of lung inflammation, including histopathological

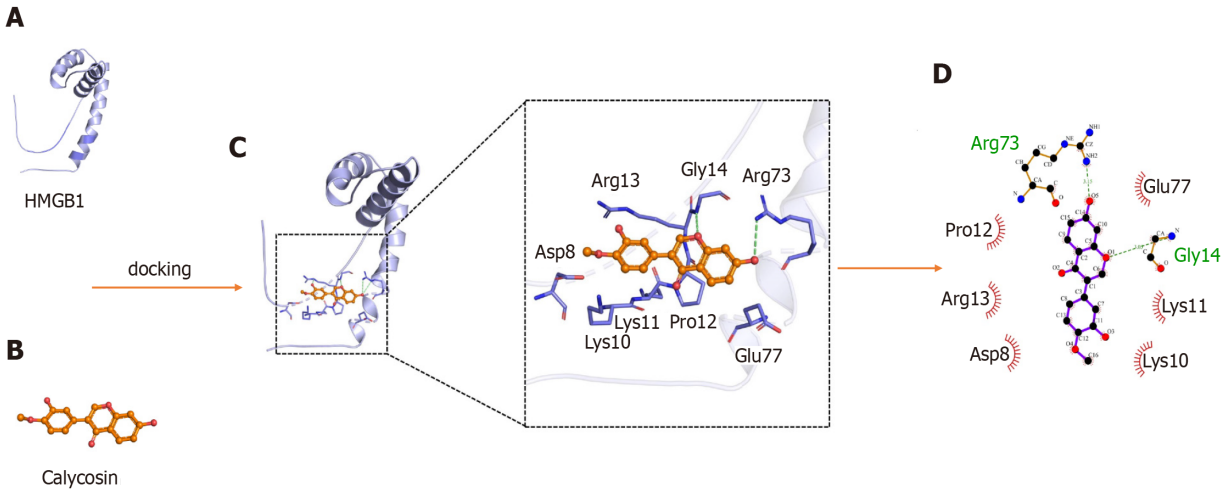

Figure 10 Theoretical binding mode of calycosin and high mobility group box 1 A-box. A: The 3D structure of high mobility group box 1 (HMGB1) A-box; B: The 3D structure of calycosin (Cal); C: 3D docking mode between Cal and HMGB1 A-box simulated by Discovery Studio and the amino acid of the active site; D: Two-dimensional schematic interaction diagram between Cal and HMGB1 A-box, the color of the amino acid residue is drawn by interaction. HMGB1: High mobility group box 1; Cal: Calycosin.

changes such as marked alveolar wall thickening, edema (demonstrated by increased lung wet/dry ratio), and pronounced inflammatory cell infiltration particularly by neutrophils (elevated lung MPO levels) was observed in SAP mice. In contrast, mice prophylactically treated with Cal showed dose-dependent amelioration of the severity of ALI in L-arg-induced SAP, as demonstrated by significant reductions in the histopathological manifestations and serum indices of SAP and ALI.

We then investigated the protective effect of Cal on the expression of inflammatory factors and neutrophil infiltration. In fact, the severity of local and systemic organ damage is dependent on the level of pro-inflammatory cytokine production[30]. Pro-inflammatory cytokines TNF-α, IL-1β and IL-6, play critical roles in the development and progression of SAP and perpetrators of systemic inflammatory response and organ damage, including ALI[31,32]. Blockade of serum TNF-α, IL-1β and IL-6 has been shown to attenuate the disease process[33,34]. Consistent with previous findings and our histopathological observations, serum levels of TNF-α, IL-6, and IL-1β were elevated in SAP mice when compared with controls as well as the mRNA levels in lung tissue, while Cal treatment reversed the change. In particular, the infiltration of inflammatory cells, mainly neutrophils, is a hallmark of tissue injury in SAP. Previous studies have demonstrated that HMGB1 and chemokine (CXC motif) ligand 1 (CXCL-1) play a role in the recruitment of neutrophils to the lungs, leading to tissue damage [8,35]. Functional inhibition of HMGB1 or CXC chemokines was shown to ameliorate tissue damage[11,12,36]. Similarly, we showed that Cal treatment suppressed the serum levels and gene expression of HMGB1 and CXCL-1 levels in the lung, with an associated reduction in neutrophil infiltration and MPO expression in lung tissue. This result may have partially contributed to the inhibitory effects of Cal on inflammatory factors and neutrophil infiltration.

Mechanistically, the HMGB1-dependent activation of NF-κB has been implicated in the development of ALI[37]. Studies have shown that the suppression of HMGB1 expression using siRNA can inhibit NF-κB activation, reduce inflammatory reactions, and protect mice against developing ALI in SAP[38,39]. Consistent with these reports, we demonstrated that Cal treatment dose-dependently inhibited the expression of HMGB1 and NF-κB signaling activation both *in vivo* and *in vitro*. In addition, studies have illustrated that HMGB1 mediates pancreatic pain by targeting RAGE and the CXCL12/CXCR4 signaling axis in mice with AP[40], therefore, pain may be relieved in animals after Cal treatment.

This study provides an experimental basis for the clinical application of Cal, which may be a candidate for the treatment of SAP-ALI patients in the future. However, there are limitations to the present study. For example, Cal inhibited the HMGB1/NF-κB signaling pathway *in vivo* and *in vitro* and validated the interaction through molecular docking. Therefore, the specific interaction between Cal and HMGB1 requires further study.

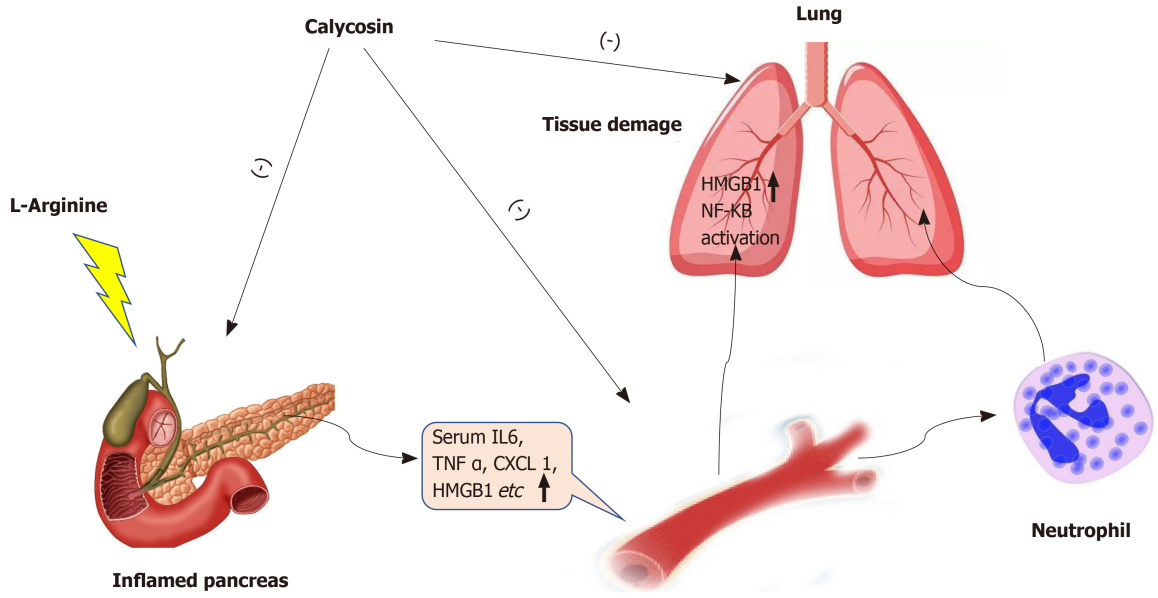

Figure 11 Calycosin attenuates acute lung injury in L-arginine induced severe acute pancreatitis by curtailing high mobility group box 1-induced inflammation.

CONCLUSION

In summary, our data clearly demonstrated that Cal exhibits protective and beneficial effects against ALI in SAP by averting local and systemic neutrophil infiltration and the inflammatory response in part *via* the suppression of HMGB1-NF-κB signaling activation. (Figure 11).

ARTICLE HIGHLIGHTS

Research background

Acute lung injury (ALI) is a common and life-threatening complication of severe acute pancreatitis (SAP). There are currently limited effective treatment options for SAP and associated ALI. Calycosin (Cal), a bioactive constituent extracted from the medicinal herb *Radix astragali* exhibits potent anti-inflammatory properties, but its effect on SAP and associated ALI has yet to be determined.

Research motivation

To determine the effect of Cal on the SAP-ALI and its underlying mechanism.

Research objectives

To identify the roles of Cal in SAP-ALI and the underlying mechanism.

Research methods

SAP was induced *via* two intraperitoneal injections of L-arginine (L-arg: 4g/kg). Cal-treated mice received intraperitoneal injections of Cal (25 or 50 mg/kg) 1 h prior to the first L-arg challenge. Mice were sacrificed 72 h after the second L-arg challenge and indices of SAP and associated ALI were examined histologically and biochemically. An *in vitro* model of lipopolysaccharide (LPS)-induced ALI was established using A549 cells. Cells were either fixed for immunofluorescence analysis or protein extracted for western blot assessment of High Mobility Group Box 1(HGMB1) and nuclear factor-kappa B (NF-κB) expression, respectively. Molecular docking analyses were conducted to examine the interaction of Cal with HMGB1.

Research results

Cal treatment significantly reduced serum amylase levels and alleviated histopathological injury associated with SAP and ALI. Neutrophil infiltration and lung tissue levels of the neutrophil mediator myeloperoxidase (MPO) were reduced in line with

the protective effects of Cal against ALI in SAP. Cal treatment also attenuated the serum levels and mRNA expression of pro-inflammatory cytokines in lung tissue. Cal treatment markedly suppressed the expression of HMGB1 and phosphorylated NF-κB p65 in lung tissues and in an *in vitro* model of LPS-induced ALI in A549 cells. Furthermore, molecular docking analysis provided evidence for the direct interaction of Cal with HGMB1.

Research conclusions

Cal protects mice against L-arg-induced SAP and associated ALI by attenuating local and systemic neutrophil infiltration and the inflammatory response *via* inhibition of HGMB1 and the NF-κB signaling pathway.

Research perspectives

Cal may be used as a potential medicine in SAP-ALI therapy.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7687-7704

DOI: 10.3748/wjg.v27.i44.7687

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

ORIGINAL ARTICLE

Basic Study

Prediction of genetic alterations from gastric cancer histopathology images using a fully automated deep learning approach

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Author contributions: Jang HJ and Lee SH were responsible for the study concept and design; Lee SH enrolled the cohorts and collected clinicopathological data from patients; Lee A, Kang J, Song IH and Lee SH performed the assays; Jang HJ , Lee A, Kang J, Song IH and Lee SH analyzed data; Jang HJ and Lee SH wrote the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the College of Medicine at the Catholic University of Korea (KC19SESI0787).

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

Data sharing statement: No additional data are available.

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

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Abstract

BACKGROUND

Studies correlating specific genetic mutations and treatment response are ongoing to establish an effective treatment strategy for gastric cancer (GC). To facilitate this research, a cost- and time-effective method to analyze the mutational status is necessary. Deep learning (DL) has been successfully applied to analyze hematoxylin and eosin (H and E)-stained tissue slide images.

AIM

To test the feasibility of DL-based classifiers for the frequently occurring mutations from the H and E-stained GC tissue whole slide images (WSIs).

METHODS

From the GC dataset of The Cancer Genome Atlas (TCGA-STAD), wild-type/mutation classifiers for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes were trained on 360 × 360-pixel patches of tissue images.

RESULTS

The area under the curve (AUC) for the receiver operating characteristic (ROC) curves ranged from 0.727 to 0.862 for the TCGA frozen WSIs and 0.661 to 0.858 for the TCGA formalin-fixed paraffin-embedded (FFPE) WSIs. The performance of the classifier can be improved by adding new FFPE WSI training dataset from our institute. The classifiers trained for mutation prediction in colorectal cancer completely failed to predict the mutational status in GC, indicating that DL-based mutation classifiers are incompatible between different cancers.

CONCLUSION

Supported by the National Research Foundation of Korea funded by the Korea government (Ministry of Science and ICT), No. 2019R1F1A1062367.

Country/Territory of origin: South Korea

Specialty type: Pathology

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

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

Received: July 29, 2021
Peer-review started: July 29, 2021
First decision: August 19, 2021
Revised: September 5, 2021
Accepted: November 13, 2021
Article in press: November 13, 2021
Published online: November 28, 2021

P-Reviewer: Kumar I, Nearchou IP, Salvi M
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

This study concluded that DL could predict genetic mutations in H and E-stained tissue slides when they are trained with appropriate tissue data.

Key Words: Gastric cancer; Mutation; Deep learning; Digital pathology; Formalin-fixed paraffin-embedded

Core Tip: Recently, deep learning approach has been implemented to predict the mutational status from hematoxylin and eosin (H and E)-stained tissue images of diverse tumors. The aim of our study was to evaluate the feasibility of classifiers for mutations in the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in gastric cancer tissues. The area under the curves for receiver operating characteristic curves ranged from 0.727 to 0.862 for the The Cancer Genome Atlas (TCGA) frozen tissues and 0.661 to 0.858 for the TCGA formalin-fixed paraffin-embedded tissues. This study confirmed that deep learning-based classifiers can predict major mutations from the H and E-stained gastric cancer whole slide images when they are trained with appropriate data.

Citation: Jang HJ, Lee A, Kang J, Song IH, Lee SH. Prediction of genetic alterations from gastric cancer histopathology images using a fully automated deep learning approach. *World J Gastroenterol* 2021; 27(44): 7687-7704
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7687.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7687

INTRODUCTION

Molecular tests to identify specific mutations in solid tumors have improved our ability to stratify cancer patients for more selective treatment regimens[1]. Therefore, molecular tests to detect various mutations are recommended for some tumors, including EGFR mutations in lung cancer, KRAS in colorectal cancer, and BRAF in melanoma. However, it is not routinely applied to cancer patients because molecular tests are not cost- and time-efficient[2]. Furthermore, the clinical significance of many mutations is still not well understood. For example, mutation profiling of gastric cancer (GC) is still proceeding, and the meaning of each mutation is not clearly understood[3]. GC is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide[4]. It is important to evaluate the relationship between the mutational status and clinical characteristics of GC to improve the clinical outcomes of GC patients. Furthermore, many targeted drugs for treating various tumors are not effective in GC therapy because GC is not enriched with known driver mutations[5]. Therefore, research to characterize the roles of GC-related genes on the clinical behavior of tumors and the potential response to targeted therapies will have immense importance for the improvement of treatment response in GC[6]. A cost- and time-effective method to determine the mutational status of GC patients is necessary to promote these studies.

Recently, deep learning (DL) has been increasingly implemented to predict the mutational status from hematoxylin and eosin (H and E)-stained tissue slides of various cancers[7-11]. The H and E-stained tissue slides were made for almost all cancer patients for basic diagnostic studies by pathologists[12]. Therefore, mutation prediction from the H and E-stained tissue slide based on a computational method can be a cost- and time-effective alternative tool for conventional molecular tests[13-15]. Although it has long been recognized that the morphological features of tissue architecture reflect the underlying molecular alterations[16,17], the features are not easily identifiable by human evaluators[18,19]. DL offers an alternative solution to overcome the limitations of a visual examination of tissue morphology by pathologists. By combining feature learning and model fitting in a unified step, DL can capture the most discriminative features for a given task directly from a large set of tissue images [20]. Digitization of tissue slides has been rapidly increasing after the approval of digitized whole-slide images (WSIs) for diagnostic purposes[21]. Digitized tissue data

are rapidly accumulating with their associated mutational profiles. Therefore, the DL-based analysis of tissue slides for the mutational status of cancer tissues has immense potential as an alternative or complementary method for conventional molecular tests.

Based on the potential of DL for the detection of mutations from digitized tissue slides, in a previous study, we successfully built DL-based classifiers for the prediction of mutational status of APC, KRAS, PIK3CA, SMAD4, and TP53 genes in colorectal cancer tissue slides[11]. This study investigated the feasibility of classifiers for mutations in the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in GC tissues. First, the classifiers were trained and tested for GC tissue slides from The Cancer Genome Atlas (TCGA). The generalizability of the classifiers was tested using an external dataset. Then, new classifiers were trained for combined datasets from TCGA and external datasets to investigate the effect of the extended datasets. The results suggest that it is feasible to predict mutational status directly from tissue slides with deep learning-based classifiers. Finally, as the classifiers for KRAS, PIK3CA, and TP53 mutations for both colorectal and GC were available, we also analyzed the generalizability of the DL-based mutation classifiers trained for different cancer types.

MATERIALS AND METHODS

Part I: Tests with The Cancer Genome Atlas whole-slide image datasets

Patient cohort: The Cancer Genome Atlas (TCGA) provides extensive archives of digital pathology slides with multi-omics test results to test the possibility of tissue-based mutation detection[22]. After a carefully review of all the WSIs in the TCGA GC dataset (TCGA-STAD), we eliminated WSIs with poor scan quality and very small tumor contents. We selected slides from 25, 19, 34, 64, and 160 patients, which were confirmed to have mutations in CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. There were more than two slides for many patients in the TCGA dataset, with a maximum of four slides for some patients. However, in many cases, one or two slides contained only normal tissues. We excluded normal slides and selected a maximum of two tumor-containing slides per patient. The final number of frozen tissue slides was 34, 26, 50, 94, and 221 and that of formalin-fixed paraffin-embedded (FFPE) tissue slides was 27, 19, 34, 66, and 174 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. We selected 183 patients with wild-type CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Therefore, the same patients with wild-type genes for CDH1, ERBB2, KRAS, PIK3CA, and TP53 can be involved in the training of every classifier as a non-mutated group. This may help the comparison of the different classifiers more standardized because they all have the same group of patients as the wild-type group. The TCGA IDs of the patients in each group are listed in Supplementary Table 1. Our previous studies recognized that a DL model cannot perform optimally for both training and testing unless the dataset is forced to have similar amounts of data between classes[23]. Therefore, we limited the difference in patient numbers between the mutation and wild-type groups to less than 1.4 fold by random sampling. For example, only 35 of the 183 wild-type patients were randomly selected as the CDH1 wild-type group because there were only 25 CDH1 mutated patients. Ten-fold cross-validation was performed based on these randomly sampled wild-type patients. However, the classifiers yielded better results when the tumor patches from all wild-type patients other than the test sets were randomly sampled to match the 1.4 fold data ratio of wild-type/mutation groups for training, as this strategy could include a greater variety of tissue images. Therefore, we included all wild-type patients other than the test sets during training and randomly selected patients during testing.

Deep learning model: In general, a WSI is too large to be analyzed simultaneously using a deep neural network. Therefore, the analysis results for small image patches are integrated for conclusion. We divided a WSI into non-overlapping patches of 360 × 360 pixel tissue images at 20× magnification to detect mutational status. To make the classification process fully automated, artifacts in the WSIs such as air bubbles, compression artifacts, out-of-focus blur, pen markings, tissue folding, and white background should be removed automatically. A simple convolutional neural network (CNN), termed as tissue/non-tissue classifier, was trained to discriminate these various artifacts all at once. The structure of the tissue/non-tissue classifier was described in our previous study[11]. The tissue/non-tissue classifier could filter out almost 99.9% of the improper tissue patches. Then, tissue patches classified as "improper" by the tissue/non-tissue classifier were removed, and the remaining

"proper" tissue patches were collected. For the tumor or mutation classifiers described below, only proper tissue patches were analyzed (Figure 1).

Morphologic features reflecting mutations in specific genes might be expressed mainly in tumor tissues rather than normal tissues[24,25]. Therefore, tumor tissues should be separated from the WSI to predict the mutational status of the WSI. In a previous study, we successfully built normal/tumor classifiers for various tumors, including GC[26]. We concluded that frozen and FFPE slides should be separately analyzed using a deep neural network due to their different morphologic features. Thus, we adopted the normal/tumor classifiers for frozen and FFPE tissue slides from a previous study to delineate the normal/tumor gastric tissues for the frozen and FFPE slides of the TCGA-STAD dataset in the present study. Mutation classifiers were trained separately for the selected tumor patches for frozen and FFPE tissues. We selected tumor patches with a tumor probability higher than 0.9 to collect tissue patches with evident tumor features. We adopted a patient-level ten-fold cross-validation to completely characterize the TCGA-STAD dataset. Therefore, patients in each mutation/wild-type group for the five genes were separated into ten different folds, and one of the ten folds was used to test the classifiers trained with data from the other nine folds. Therefore, ten different classifiers were trained and tested for each group. The same label for all tumor tissue patches in a WSI as either 'wild-type' or 'mutated' were assigned based on the mutational status of the patient. Thereafter, the Inception-v3 model, a widely used CNN architecture, was trained to classify the tumor patches into 'wild-type' or 'mutated' tissues, as in our previous study on mutation prediction in colorectal cancer[11]. We fully trained the network from the beginning and did not adopt a transfer-learning scheme. The average probability of all tumor patches in a WSI was calculated to determine the slide-level mutation probability of a WSI. The Inception-v3 model was implemented using the TensorFlow DL library (http://tensorflow.org), and the network was trained with a mini-batch size of 128 and cross-entropy loss function as a loss function. For training, we used the RMSProp optimizer, with an initial learning rate of 0.1, weight decay of 0.9, momentum of 0.9, and epsilon of 1.0. Ten percent of the training slides were used as the validation dataset, and training was stopped when the loss for the validation data started to increase. Data augmentation techniques, including random horizontal/vertical flipping and random rotations by 90°, were applied to the tissue patches during training. Color normalization was applied to the tissue patches to avoid the effect of stain differences[27,28]. At least five classifiers were trained on each fold of mutation for the frozen and FFPE WSIs separately. The classifier with the best area under the curve (AUC) for the receiver operating characteristic (ROC) curves on the test dataset was included in the results. The ROC curves for fold with the lowest AUC, highest AUC, and the concatenated results for data from all ten folds are shown in the figures.

In summary, a WSI is analyzed as follows: 1. The whole slide is split into non-overlapping 360 × 360 pixel tissue patches, 2. Proper tissue patches are selected by tissue/non-tissue classifier, 3. Only tumor patches with tumor probability higher than 0.9 are selected, 4. High probability tumor patches are classified by each wild-type/mutation classifier, 5. The probabilities of tumor patches are averaged to obtain the slide-level mutation probability. The number of tissue patches used for the training of all mutation prediction models is summarized in Supplementary Table 2. The average number of training epochs for each classifier is summarized in Supplementary Table 3.

Part II: Tests on the external cohorts

Patient cohort: GC tissue slides were collected from 96 patients who had previously undergone surgical resection at Seoul St. Mary's Hospital between 2017 and 2020 (SSMH dataset). An Aperio slide scanner (Leica Biosystems) was used to scan the FFPE slides. The Institutional Review Board of the College of Medicine at the Catholic University of Korea approved this study (KC19SESI0787).

Mutation prediction on SSMH dataset: For CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, 6, 6, 12, 11, and 39 patients were confirmed to have the mutations, respectively. Thirty-eight patients had wild-type genes for all five genes. For CDH1, ERBB2, KRAS, and PIK3CA genes, we selected the number of wild-type patients to be 1.4 times that of mutated patients. For TP53, all 38 patients with wild-type genes were enrolled. The normal/tumor classifier for TCGA FFPE tissues was also used to discriminate the tumor tissue patches of SSMH WSIs. Our previous study showed that the normal/tumor classifier for TCGA-STAD was valid for SSMH FFPE slides[29]. First, the mutational status of the SSMH slides was analyzed by classifiers trained on TCGA-

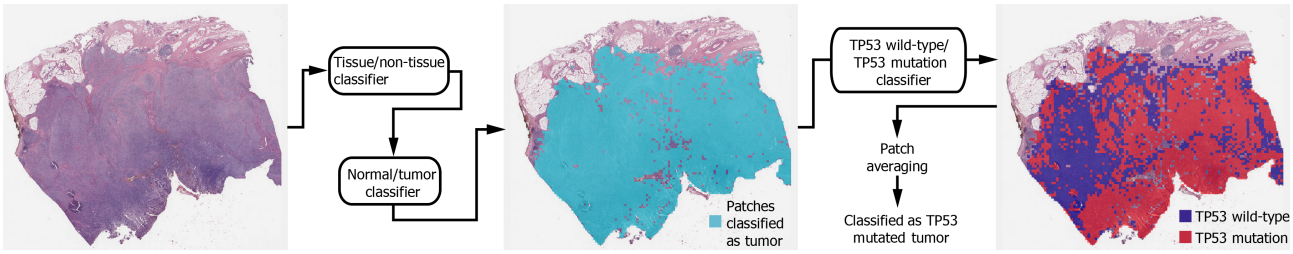


Figure 1 Workflow for the fully automated prediction of mutation. Tissue image patches with tumor probability higher than 0.9 were selected by sequential application of the tissue/non-tissue and normal/tumor classifiers. Then the tumor patches were classified into the wild-type or mutated patches. The patch-level probabilities of mutation are averaged to yield the slide-level probability.

STAD FFPE WSIs. Subsequently, new classifiers were trained using both TCGA and SSMH FFPE tissues. Patient-level three-fold cross validation was applied to the SSMH datasets because the number of mutated patients was not sufficient for ten-fold cross-validation.

Statistical analysis

To demonstrate the performance of each classifier, the ROC curves and their AUCs are presented in the figures. For the concatenated results from all ten folds, 95% confidence intervals (CIs) were also presented using the percentile bootstrap method. In addition, the accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models with cutoff values for maximal Youden index (sensitivity + specificity - 1) were presented. We used a permutation test with 1000 iterations to compare the differences between the two paired or unpaired ROC curves when a comparison was necessary[30]. Statistical significance was set at $P < 0.05$.

RESULTS

Tissue patches with high tumor probability were automatically collected from a WSI by sequentially applying the tissue/non-tissue and normal/tumor classifiers to 360 × 360 pixels tissue image patches (Figure 1). Then, classifiers to distinguish the mutational status of CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in the tumor tissue patches from the TCGA-STAD frozen and FFPE WSI datasets were separately trained with a patient-level ten-fold cross-validation scheme.

The classification results of the TCGA-STAD WSIs are presented in Figures 2 to 6 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Results for the frozen and FFPE tissues are presented in the upper and lower part of each figure, respectively. Panels A and C demonstrated the representative binary heatmaps of tissue patches classified as wild-type or mutated tissues. The WSIs with gene mutation correctly classified as mutation, with wild-type gene correctly classified as wild-type, with gene mutation falsely classified as wild-type, and with wild-type gene falsely classified as mutation are presented from left to right for panels A and C. The binary heatmaps were drawn with the wild-type/mutation discrimination threshold set to 0.5. We simply set the threshold to 0.5, because every classifier for different folds had different optimal thresholds. Slide-level ROC curves for folds with the lowest and highest AUCs are presented to demonstrate the differences in the performance between folds (left and middle ROC curves in each figure). Finally, the slide-level ROC curves for the concatenated results from all ten folds were used to infer the overall performance (right ROC curves). The results for the CDH1 gene are shown in Figure 2. The AUCs per fold ranged from 0.833 to 1.000 for frozen WSIs and from 0.833 to 1.000 for FFPE WSIs. The AUCs for the concatenated results were 0.842 (95%CI: 0.749-0.936) and 0.781 (95%CI: 0.645-0.917) for frozen and FFPE WSIs, respectively. For ERBB2 (Figure 3), the lowest and highest AUCs per fold were 0.667 and 1.000, respectively, for both frozen and FFPE WSIs. The concatenated AUCs were 0.751 (95%CI: 0.631-0.871) and 0.661 (95%CI: 0.501-0.821), respectively. For the KRAS gene (Figure 4), the AUCs per fold were between 0.775 and 1.000 for frozen WSIs and between 0.750 and 1.000 for FFPE WSIs. The concatenated AUCs were 0.793 (95%CI: 0.706-0.879) and 0.858 (95%CI: 0.738-0.979) for frozen and FFPE WSIs, respectively. For the PIK3CA gene (Figure 5), the concatenated AUC for the frozen WSIs was 0.862 (95%CI: 0.809-0.916), with a range of 0.705

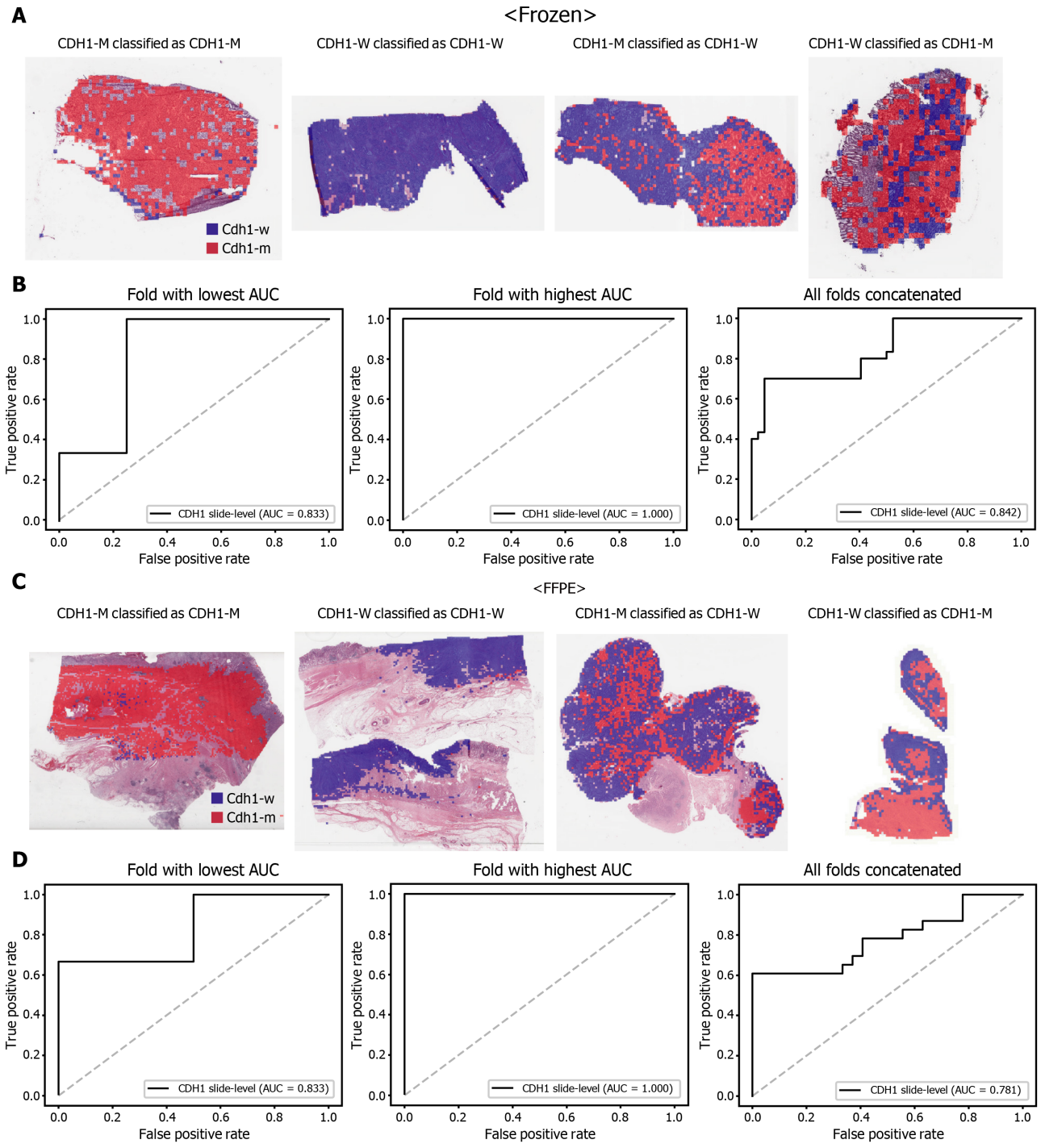


Figure 2 Classification results of CDH1 gene in the The Cancer Genome Atlas gastric cancer dataset. A: Representative whole slide images (WSIs) of the frozen slides with CDH1 gene mutation correctly classified as mutation, with wild-type gene correctly classified as wild-type, with gene mutation falsely classified as wild-type, and with wild-type gene falsely classified as mutation, from left to right; B: Receiver operating characteristic (ROC) curves for the fold with lowest area under the curve (AUC), for the fold with highest AUC, and for the concatenated results of all ten folds, from left to right, obtained with the classifiers trained with the frozen tissues; C and D: Same as A and B but the results were for the formalin-fixed paraffin-embedded WSIs. CDH1-M: CDH1 mutated, CDH1-W: CDH1 wild-type.

to 0.990. For FFPE WSIs, the lowest and highest AUCs per fold were 0.675 and 1.000, respectively, yielding a concatenated AUC of 0.828 (95%CI: 0.750-0.907). Lastly, the results for the TP53 gene are presented in Figure 6. The AUCs per fold were between 0.666 to 0.810 for frozen WSIs and between 0.702 to 0.847 for FFPE WSIs. The concatenated AUCs were 0.727 (95%CI: 0.683-0.771) and 0.727 (95%CI: 0.671-0.784) for frozen and FFPE WSIs, respectively. For the colorectal cancer dataset from TCGA, mutation classification results for frozen tissues were better than those for FFPE tissues in some genes[11]. However, there were no significant differences between the frozen and FFPE tissues in the TCGA-STAD dataset (P = 0.491, 0.431, 0.187, 0.321, and 0.613

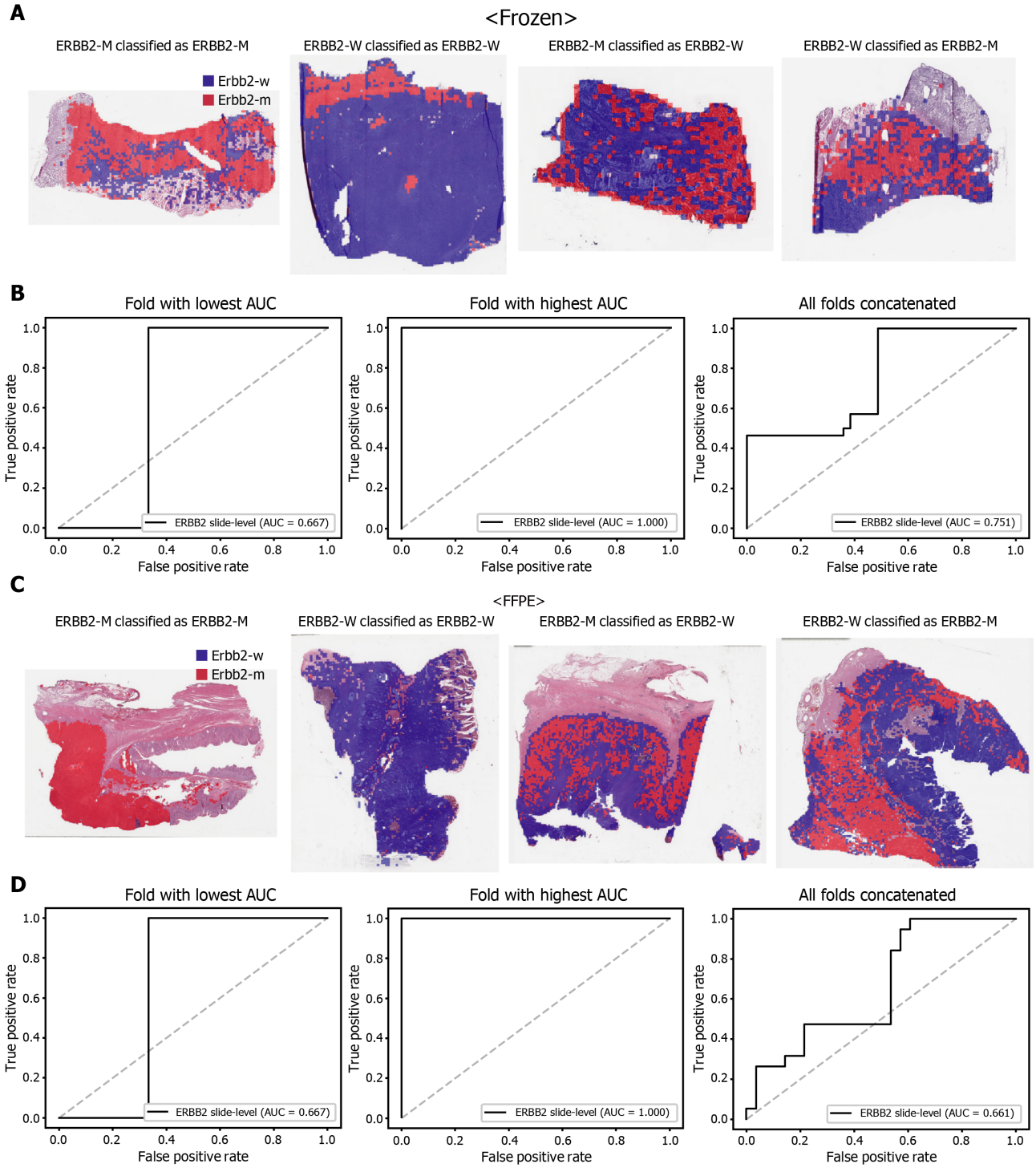


Figure 3 Classification results of ERBB2 gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. ERBB2-M: ERBB2 mutated, ERBB2-W: ERBB2 wild-type.

between the concatenated AUCs for the frozen and FFPE tissues by Venkatraman's permutation test for unpaired ROC curves for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. For a clearer assessment of the performance of each model, the accuracy, sensitivity, specificity, and F1 score of the classification results are presented in Table 1.

The performance of a DL model on an external dataset should be tested to validate the generalizability of the trained model. Therefore, we collected GC FFPE WSIs with matching mutation data from Seoul St. Mary's Hospital (SSMH dataset). The normal/tumor classifier for TCGA-STAD FFPE tissues was also applied to select tissue patches with high tumor probabilities. Thereafter, the mutation classifier for each gene

Table 1 Accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models for the The Cancer Genome Atlas datasets

	Accuracy	Sensitivity	Specificity	F1 score
TCGA Frozen Tissue Slides				
CDH1	0.847	0.700	0.952	0.792
ERBB2	0.716	1.000	0.512	0.746
KRAS	0.773	0.883	0.708	0.745
PIK3CA	0.834	0.771	0.884	0.806
TP53	0.667	0.743	0.602	0.673
TCGA FFPE Tissue Slides				
CDH1	0.820	0.608	1.000	0.756
ERBB2	0.574	1.000	0.285	0.655
KRAS	0.894	0.727	1.000	0.842
PIK3CA	0.803	0.629	0.923	0.723
TP53	0.673	0.678	0.668	0.659

TCGA: The Cancer Genome Atlas; FFPE: Formalin-fixed paraffin-embedded.

trained on the TCGA-STAD FFPE tissues was tested on the SSMH dataset. The slide-level ROC curves for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes are presented in Supplementary Figure 1. The AUCs for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes were 0.667, 0.630, 0.657, 0.688, and 0.572, respectively. For the KRAS, PIK3CA, and TP53 genes, the performance of the TCGA-trained mutation classifiers on the SSMH dataset were worse than that of the TCGA dataset ($P = 0.389$, $P = 0.849$, $P < 0.05$, $P < 0.05$, and $P < 0.05$ for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively, by Venkatraman's permutation test for unpaired ROC curves). These results demonstrate that the mutation classifiers trained with TCGA-STAD WSI datasets had limited generalizability. It is of interest if the performance can be enhanced by training the classifiers with expanded datasets, including both TCGA and SSMH datasets. Cancer tissues from different ethnic groups can show different features[16,19]; therefore, the performance of the classifiers can be improved by mixing the datasets. When the classifiers trained with the mixed datasets were used, the performance on the SSMH dataset was generally improved because the SSMH data were included in the training data in this setting (Figures 7 and 8). The AUCs became 0.778, 0.833, 0.838, 0.761, and 0.775 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively ($P = 0.234$, $P < 0.05$, $P < 0.05$, $P = 0.217$, and $P < 0.05$ between the ROCs of classification results by classifiers trained on the TCGA-STAD dataset and mixed dataset for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively, by Venkatraman's permutation test for paired ROC curves). Furthermore, the performance on the TCGA-STAD FFPE dataset was also generally improved by the new classifiers trained on both datasets, except for the PIK3CA gene, which showed worse results (Supplementary Figure 2). The AUCs were 0.918, 0.872, 0.885, 0.766, and 0.820 for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively ($P < 0.05$, $P < 0.05$, $P = 0.216$, $P < 0.05$, and $P < 0.05$ compared with the TCGA-trained classifiers by Venkatraman's permutation test for paired ROC curves). The accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models trained with both SSMH and TCGA datasets are presented in Supplementary Table 4.

Another interesting question is whether the DL-based classifiers for mutational status can be compatible with other types of cancers. We already built the mutation classifiers for KRAS, PIK3CA, and TP53 genes in the colorectal cancer dataset of TCGA in a previous study[11]. Therefore, we tested whether the classifiers trained on colorectal cancer can distinguish the mutational status in GC. As shown in Figure 9, the classifiers trained to discriminate the mutational status of KRAS, PIK3CA, and TP53 genes in the FFPE tissues of colorectal cancer approximately failed to distinguish the mutational status in the FFPE tissues of the TCGA-STAD dataset, with AUCs of 0.458, 0.550, and 0.538 for the KRAS, PIK3CA, and TP53 genes, respectively. The results indicate that the tissue morphologic features reflecting the wild-type and

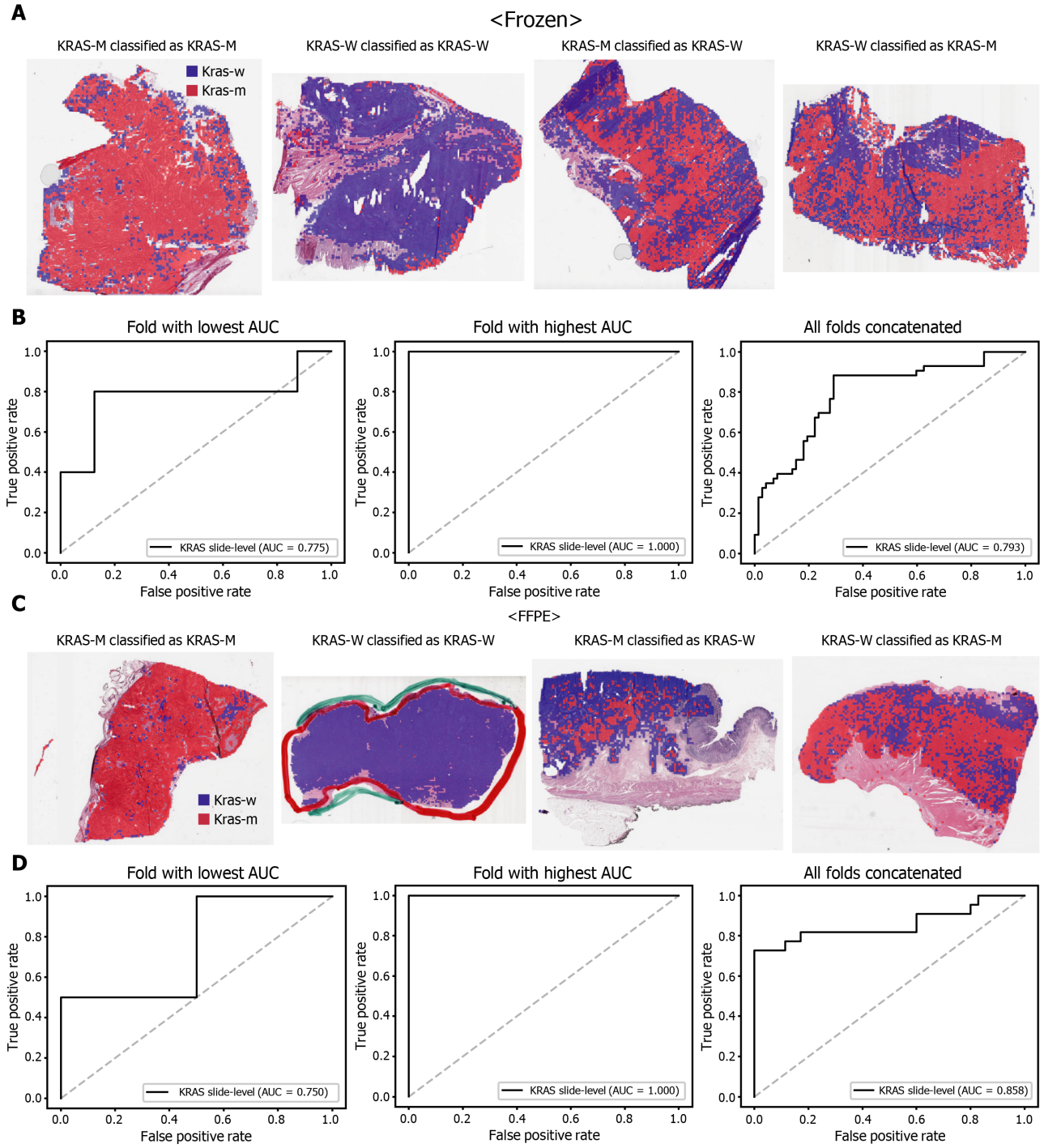


Figure 4 Classification results of KRAS gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. KRAS-M: KRAS mutated, KRAS-W: KRAS wild-type.

mutated genes are relatively different between cancers originating from different organs.

DISCUSSION

Recently, many drugs targeting specific biological molecules have been introduced to improve the survival of patients with advanced GC[31]. However, patient stratification strategies to maximize the treatment response of these new drugs are not yet well established. Targeted therapies can yield different responses depending on the mutational status of genes in patients with cancer[32]. To overcome this complexity,

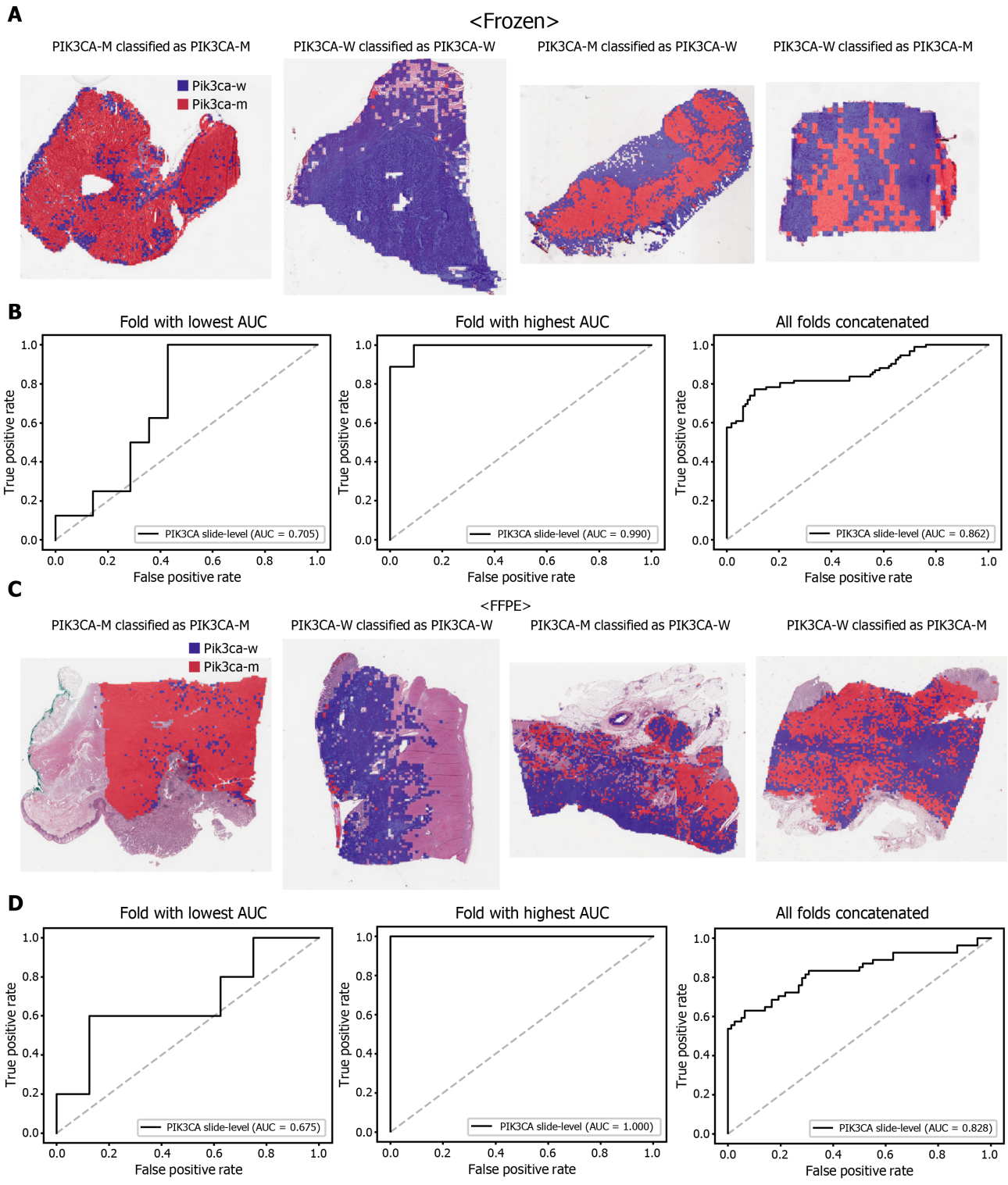


Figure 5 Classification results of PIK3CA gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. PIK3CA-M: PIK3CA mutated, PIK3CA-W: PIK3CA wild-type.

clinical trials for new drugs have begun to adopt the umbrella platform strategy, which assigns treatment arms based on the mutational status of cancer patients[6,33]. Therefore, data regarding the mutational status of cancer patients is essential for patient stratification in modern-day medicine. However, molecular tests to detect gene mutations are still not affordable for all cancer patients. If cost- and time-effective alternative methods for mutation detection can be introduced, it will promote prospective clinical trials and retrospective studies to correlate the treatment response with the mutational profiles of cancer patients, which can be retrospectively obtained from clinical data and stored tissue samples. Therefore, the new cost- and time-

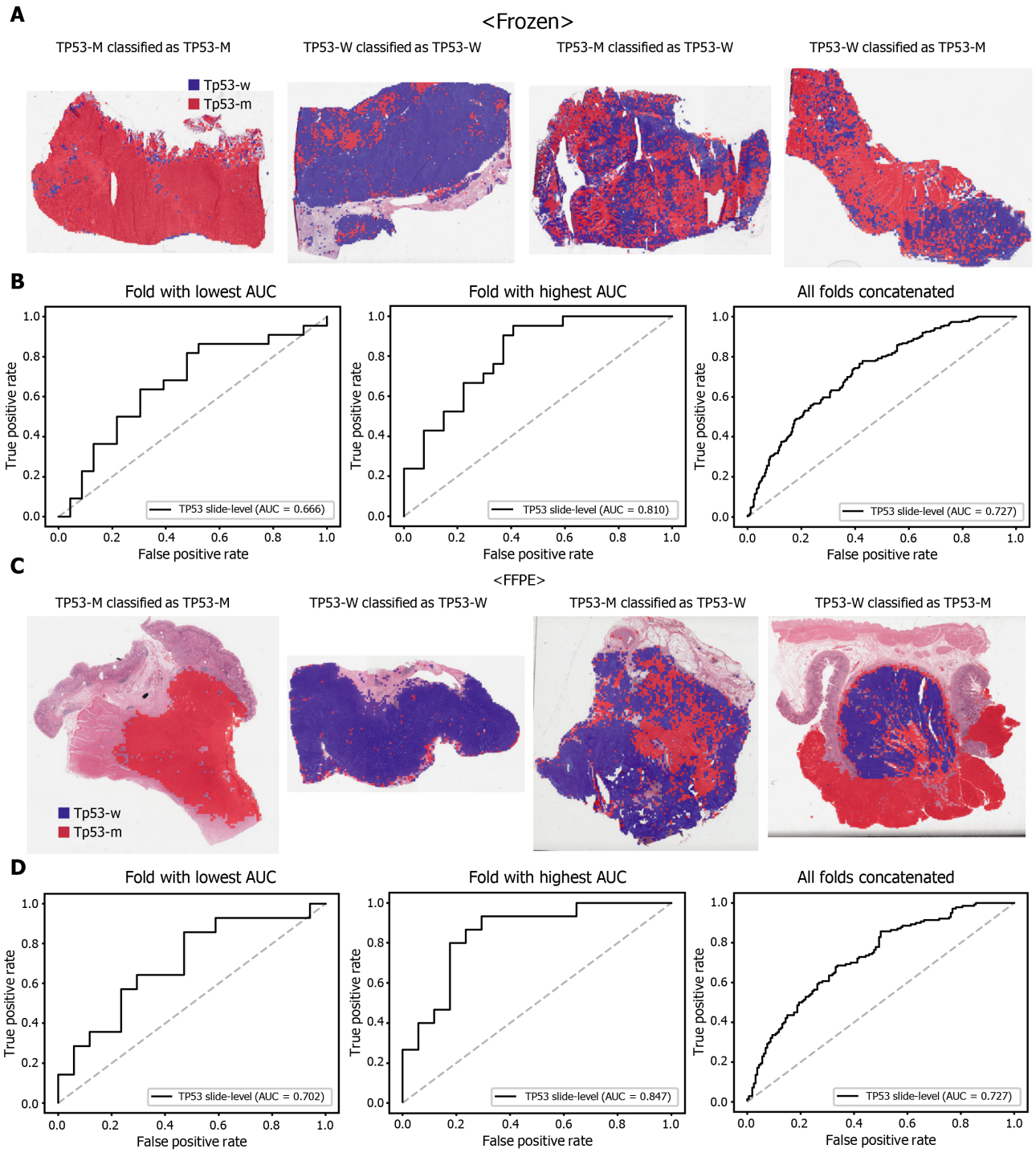
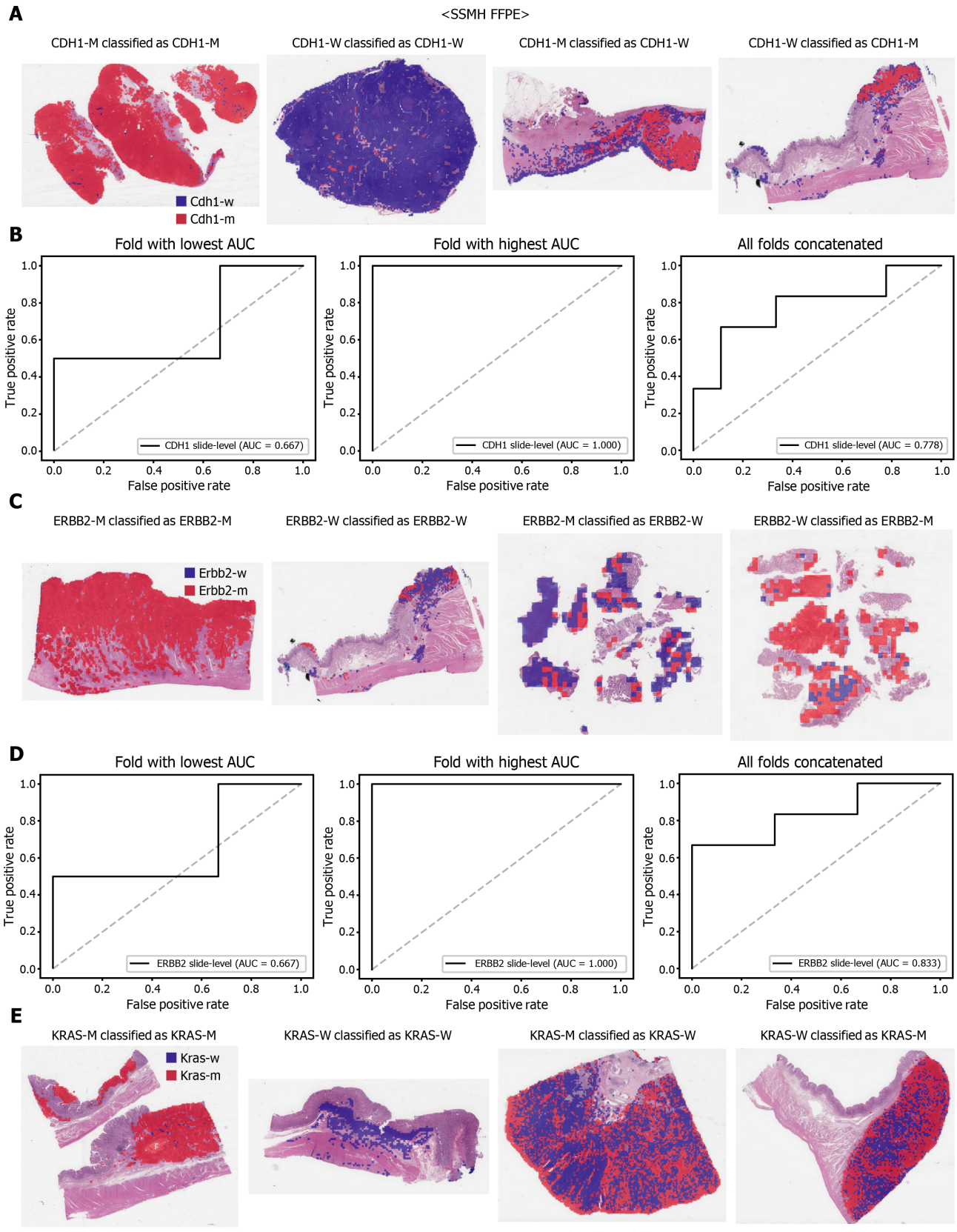


Figure 6 Classification results of TP53 gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. TP53-M: TP53 mutated, TP53-W: TP53 wild-type.

effective methods will help to establish molecular stratification of cancer patients that can be used to determine effective treatment and improve clinical outcomes[34].

Cancer tissue slides are made and stored for most cancer patients. As a result, DL-based mutation prediction from the tissue slides can be a good candidate for alternative methods. It has been well recognized that the molecular alterations are manifested as morphologic changes in tissue architecture[35]. For example, some morphological features in GC tissues have been associated with specific mutations, including CDH1 and KRAS genes[36,37]. Although it is impractical to quantitatively assess these features for the detection of mutations by visual inspection, DL can learn and distinguish subtle discriminative features for mutation detection in various cancer tissues[7-11]. This study demonstrated the feasibility of DL-based prediction of


A
<SSMH FFPE>
CDH1-M classified as CDH1-M
CDH1-W classified as CDH1-W
CDH1-M classified as CDH1-W
CDH1-W classified as CDH1-M
Cdh1-w
Cdh1-m
B
Fold with lowest AUC
Fold with highest AUC
All folds concatenated
True positive rate
False positive rate
CDH1 slide-level (AUC = 0.667)
CDH1 slide-level (AUC = 1.000)
CDH1 slide-level (AUC = 0.778)
C
ERBB2-M classified as ERBB2-M
ERBB2-W classified as ERBB2-W
ERBB2-M classified as ERBB2-W
ERBB2-W classified as ERBB2-M
Erbb2-w
Erbb2-m
D
Fold with lowest AUC
Fold with highest AUC
All folds concatenated
True positive rate
False positive rate
ERBB2 slide-level (AUC = 0.667)
ERBB2 slide-level (AUC = 1.000)
ERBB2 slide-level (AUC = 0.833)
E
KRAS-M classified as KRAS-M
KRAS-W classified as KRAS-W
KRAS-M classified as KRAS-W
KRAS-W classified as KRAS-M
Kras-w
Kras-m

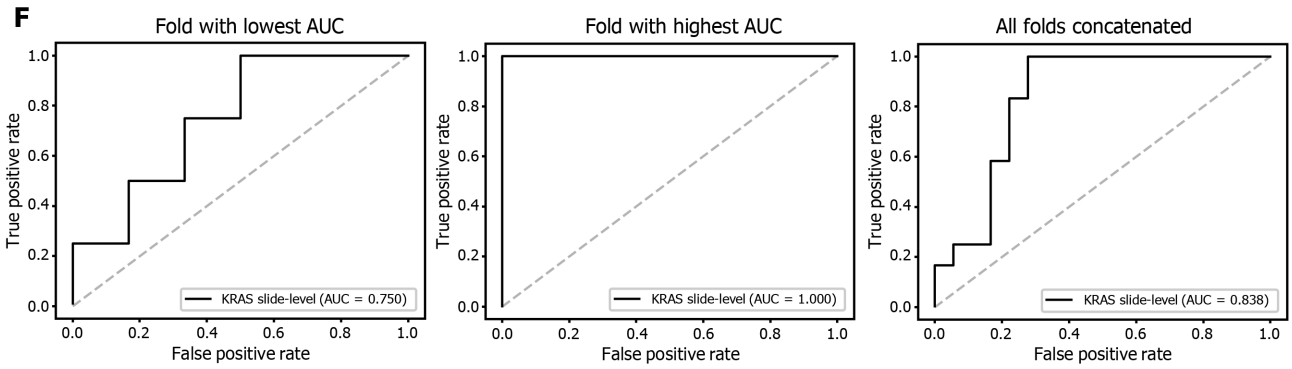


Figure 7 The classifiers trained with both The Cancer Genome Atlas and SSMH data were used to predict the mutation of CDH1 (A and B), ERBB2 (C and D), and KRAS (E and F) genes. Representative binary heatmaps of the whole slide images (WSIs) correctly classified as mutation, correctly classified as wild-type, falsely classified as wild-type, and falsely classified as mutation were presented. Receiver operating characteristic curves for the folds with the lowest and highest area under the curve and the concatenated ten folds were also presented for each gene. CDH1-M: CDH1 mutated, CDH1-W: CDH1 wild-type, ERBB2-M: ERBB2 mutated, ERBB2-W: ERBB2 wild-type, KRAS-M: KRAS mutated, KRAS-W: KRAS wild-type.

mutations in CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, which are prevalent in both the TCGA and SSMH GC datasets, from tissue slide images of GC. Other studies have also shown that mutations in these genes are frequently observed in GC[3,5]. Furthermore, many studies have attempted to evaluate the prognostic value of these mutations[3,5,38]. However, the clinical relevance of these mutations for prognosis and treatment response has not been completely determined because the studies often presented discordant results. Various factors, including the relatively low incidence of mutation, small study size, and ethnicity of the studied groups, may have contributed to the inconsistent study results. Although it is still unclear how specific mutations are involved in the prognosis and treatment response in GC patients, further studies for the fine molecular stratification of patients based on mutational status are ongoing[6]. DL-based mutation prediction from the tissue slides could provide valuable tools to support these efforts because the mutational status can be promptly obtained with minimal cost from the existing H and E-stained tissue slides.

Furthermore, DL-based classifiers can provide important information for the study of tumor heterogeneity[39]. The heatmaps of classification results overlaid on the tissue images in figures showed that mutated and wild-type regions are aggregated into separated regions. For example, the rightmost tissues in Figure 6C showed clear demarcation between TP53-mutated and wild-type regions. These results indicated that a tumor tissue can contain molecularly heterogeneous regions which can be easily visualized with the help of DL-based classifiers. The clear demarcation of molecularly heterogeneous regions in a tissue slide is an important advantage of a DL-based system and it can help the studies for the understanding of the prognostic and therapeutic values of the tumor heterogeneity without the application of the very expensive molecular tests such as multi-point single-cell sequencing.

However, further studies are needed to build practical DL classifiers for mutation prediction. Our data showed that the performance was still unsatisfactory for verifying mutational status. The AUCs ranged from 0.661 to 0.862 for the TCGA dataset. The frequency of mutation in GC TCGA dataset was relatively low. The average mutation rate for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes was 8.28%. In our previous study for the mutation prediction in colorectal cancer TCGA dataset, the average mutation rate for APC, KRAS, PIK3CA, SMAD4, and TP53 genes was 39.18%[11]. Furthermore, the classifiers showed limited generalizability to the external dataset. Because DL critically depends on data for learning prominent features, it is generally recommended to build a large multinational dataset[1,2]. Therefore, to test whether the expanded dataset can improve the performance of the classifiers, new classifiers were trained using mixed data from the TCGA and SSMH datasets. As a result, the AUCs generally increased with the larger multinational datasets. These results suggest that we could improve the performance of DL-based mutation classifiers if a large multi-national and multi-institutional dataset can be built. One exception was the PIK3CA gene, which showed worse performance for the TCGA FFPE slides by a classifier trained with the mixed dataset. Although the reason for the decreased performance is unclear, we speculate that there are some different tissue features for the wild-type and mutated PIK3CA gene between the two datasets due to different ethnicities, which could negatively affect the feature learning process for the

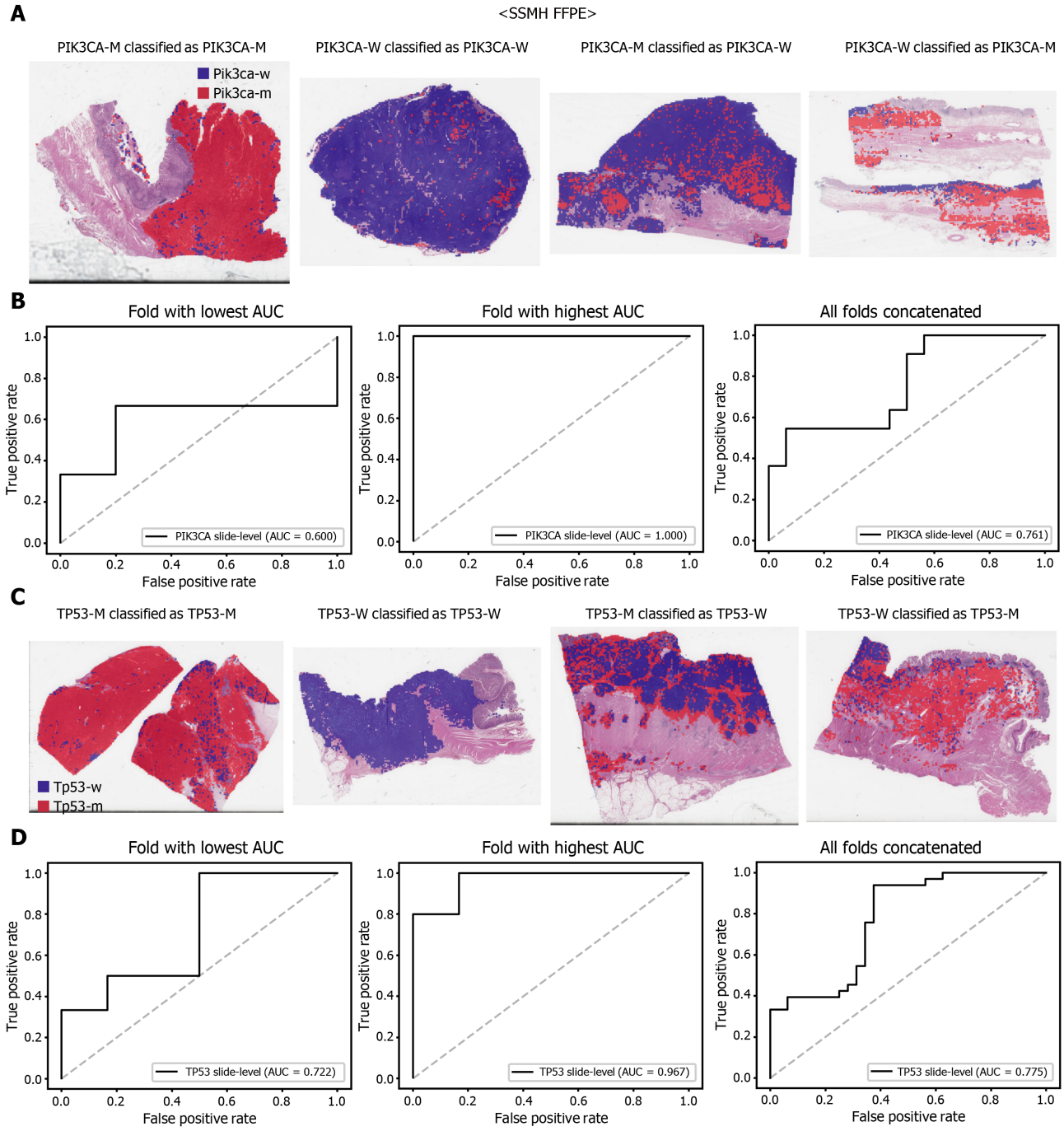


Figure 8 Mutation prediction of PIK3CA (A and B) and TP53 (C and D) genes for the SSMH gastric cancer tissue slides by the classifiers trained with both The Cancer Genome Atlas and SSMH data. The configuration of the figure is the same as in figure 7. PIK3CA-M: PIK3CA mutated, PIK3CA-W: PIK3CA wild-type, TP53-M: TP53 mutated, TP53-W: TP53 wild-type.

TCGA dataset. In addition, the numbers of patients with PIK3CA mutations were different; 64 and 11 for the TCGA and SSMH datasets, respectively. The different numbers of patients also hamper proper feature learning for the mixed dataset because data imbalance usually negatively affects the learning process. Furthermore, the studied tissues carry many additional mutations other than CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Because every tissue presented a different combination of mutations, the confounding effect of a mixture of different mutations on the tissue morphology would hamper the effective learning of features for the selected mutation. This factor also necessitates larger tissue datasets for proper learning of morphological features of specific mutations, irrespective of coexisting mutations. In our opinion, the datasets are still immature for building a prominent classifier for mutation prediction. Therefore, efforts to establish a larger tissue dataset with a mutation profile will help to understand the potential of DL-based mutation prediction systems. Recently, many

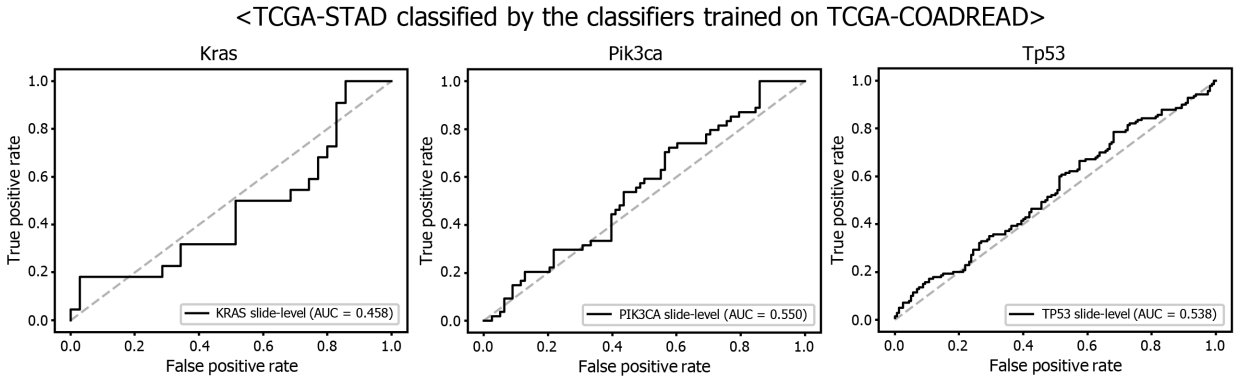


Figure 9 Mutation prediction of KRAS, PIK3CA, and TP53 genes for the The Cancer Genome Atlas gastric cancer tissue slides by the classifiers trained with The Cancer Genome Atlas colorectal cancer tissues. Receiver operating characteristic curves of the classification results for each gene were presented.

countries have started to build nationwide datasets of pathologic tissue WSIs with genomic information. Therefore, we expect that the performance of DL-based mutation prediction can be greatly improved.

Although we argued for the potential of DL-based mutation classifiers, there are important barriers to the adoption of DL-based assistant systems. First, the 'black box' nature of DL limits the interpretability of DL models and remains a significant barrier in their validation and adoption in clinics[18,40,41]. We could not trust a decision made by a DL model before we could clearly understand the basis of the decision. Therefore, a method for visualizing the features that determine the behavior of a DL model should be developed. Another barrier is the need for an individual DL system for an individual task. As described, separate systems should be built for tasks such as the classification of normal/tumor tissues for frozen and FFPE tissues. The classifier for each mutation should also be built separately. Furthermore, as shown in Figure 9, there was no compatibility between the different cancer types for the classification of genetic mutations. Therefore, many classifiers should be built to achieve optimal performance. It requires time to build many necessary classifiers to renovate current pathology workflows.

CONCLUSION

Despite these limitations, DL has enormous potential for innovative medical practice. It can help capture important information by learning features automatically from the data that are waiting to be explored in the vast database of modern hospital information systems. This information will be used to determine the best medical practice and improve patient outcomes. The tissue slides of cancer patients contain important information on the prognosis of patients[42]; therefore, DL-based analysis of tissue slides has enormous potential for fine patient stratification in the era of precision medicine. Furthermore, its cost- and time-effective nature could help save the medical cost and decision time for patient care.

ARTICLE HIGHLIGHTS

Research background

Studies correlating specific genetic mutations and treatment response are ongoing to establish an effective treatment strategy for gastric cancer (GC). With the increased digitization of pathologic tissue slides, deep learning (DL) can be a cost- and time-effective method to analyze the mutational status directly from the hematoxylin and eosin (H and E)-stained tissue whole slide images (WSIs).

Research motivation

Recent studies suggested that mutational status can be predicted directly from the H and E-stained WSIs with DL-based methods. Motivated by these studies, we invest-

igated the feasibility of DL-based mutation prediction for the frequently occurring mutations from H and E-stained WSIs of GC tissues.

Research objectives

To predict the mutational status of CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes from the H and E-stained WSIs of GC tissues with DL-based methods.

Research methods

DL-based classifiers for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 mutations were trained for the The Cancer Genome Atlas (TCGA) datasets. Then, the classifiers were validated with our own dataset. Finally, TCGA and our own dataset were combined to train a new classifier to test the effect of extended data on the performance of the classifiers.

Research results

The area under the curve (AUC) for receiver operating characteristic (ROC) curves were between 0.727 and 0.862 for the TCGA frozen WSIs and between 0.661 and 0.858 for the TCGA formalin-fixed paraffin-embedded WSIs. Furthermore, the results could be improved with the classifiers trained with both TCGA and our own dataset.

Research conclusions

This study demonstrated that mutational status could be predicted directly from the H and E-stained WSIs of GC tissues with DL-based methods. The performance of the classifiers could be improved if more data can be used to train the classifiers.

Research perspectives

Current molecular tests for the mutational status are not feasible for all cancer patients because of technical barriers and high costs. Although there is still room for much improvement, the DL-based method can be a reasonable alternative for molecular tests. It could help to stratify patients based on their mutational status for retrospective studies or prospective clinical trials with very low cost. Furthermore, it could support the decision-making process for the management of patients with GCs.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7705-7715

DOI: 10.3748/wjg.v27.i44.7705

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

ORIGINAL ARTICLE

Observational Study

Autosomal recessive 333 base pair interleukin 10 receptor alpha subunit deletion in very early-onset inflammatory bowel disease

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Author contributions: Lv JJ and Su W contributed equally to this work; Lv JJ and Su W performed the experiments, data acquisition, analysis and interpretation, and drafting of the article; Chen XY, Yu Y, Xu X, Xu CD, Deng X, and Huang JB analyzed the data and critically revised the article; Wang XQ and Xiao Y designed the project and critically revised the article for important intellectual content; and all authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Ruijin Hospital (Shanghai).

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

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Abstract

BACKGROUND

Interleukin 10 receptor alpha subunit (IL10RA) dysfunction is the main cause of very early-onset inflammatory bowel disease (VEO-IBD) in East Asians.

AIM

To identify disease-causing gene mutations in four patients with VEO-IBD and verify functional changes related to the disease-causing mutations.

METHODS

From May 2016 to September 2020, four young patients with clinically diagnosed VEO-IBD were recruited. Before hospitalization, using targeted gene panel sequencing and trio-whole-exome sequencing (WES), three patients were found to harbor a *IL10RA* mutation (c.301C>T, p.R101W in one patient; c.537G>A, p.T179T in two patients), but WES results of the fourth patient were not conclusive. We performed whole-genome sequencing (WGS) on patients A and B and reanalyzed the data from patients C and D. Peripheral blood mononuclear cells (PBMCs) from patient D were isolated and stimulated with lipopolysaccharide (LPS), interleukin 10 (IL-10), and LPS + IL-10. Serum IL-10 levels in four patients and tumor necrosis factor-α (TNF-α) in the cell supernatant were determined by enzyme-linked immunosorbent assay. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Tyr705 and Ser727 in PBMCs was determined by western blot analysis.

RESULTS

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

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.

Supported by the National Natural Science Foundation of China, No. 81741103.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

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

Received: May 31, 2021
Peer-review started: May 31, 2021
First decision: July 1, 2021
Revised: July 9, 2021
Accepted: November 9, 2021
Article in press: November 9, 2021
Published online: November 28, 2021

P-Reviewer: Nayudu SK

The four children in our study consisted of two males and two females. The age at disease onset ranged from 18 d to 9 mo. After hospitalization, a novel 333-bp deletion encompassing exon 1 of *IL10RA* was found in patients A and B using WGS and was found in patients C and D after reanalysis of their WES data. Patient D was homozygous for the 333 bp deletion. All four patients had elevated serum IL-10 levels. *In vitro*, IL-10-stimulated PBMCs from patient D failed to induce STAT3 phosphorylation at Tyr705 and only minimally suppressed TNF-α production induced by LPS. Phosphorylation at Ser727 in PBMCs was not affected by LPS or LPS + IL-10 in both healthy subjects and in patient D.

CONCLUSION
WGS revealed a novel 333-bp deletion of *IL10RA* in four patients with VEO-IBD, whereas the WES results were inconclusive.

Key Words: Interleukin 10 receptor alpha subunit mutation; Very early-onset inflammatory bowel disease; Whole-genome sequencing; Immunodeficiency; Crohn's disease; Whole-exon sequencing

Core Tip: Children less than 6 years old with very early-onset inflammatory bowel disease (VEO-IBD) exhibit severe and refractory disease phenotypes, which indicate a monogenic type disease. Here, we report four cases clinically diagnosed with VEO Crohn's disease, of which three were compound heterozygous carriers for a 333-bp deletion and an additional single-nucleotide variant, and one was homozygous for the 333-bp deletion in *IL10RA*. Based on these cases with heterozygous pathogenic variants in *IL10RA*, the possibility of another large fragment deletion that can be missed by whole-exon sequencing or gene panels should be considered, particularly when serum IL-10 is increased in patients with VEO-IBD.

Citation: Lv JJ, Su W, Chen XY, Yu Y, Xu X, Xu CD, Deng X, Huang JB, Wang XQ, Xiao Y. Autosomal recessive 333 base pair interleukin 10 receptor alpha subunit deletion in very early-onset inflammatory bowel disease. *World J Gastroenterol* 2021; 27(44): 7705-7715
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7705.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7705

INTRODUCTION

Inflammatory bowel disease (IBD) in children < 6 years of age is known as very early-onset IBD (VEO-IBD)[1] and represents a specific disease course with a distinct phenotype that can be more severe and refractory than classic IBD[2-3]. Recent studies suggested that patients with VEO-IBD, particularly those with symptoms such as perianal disease soon after birth, suffer from failed treatment, indicating a monogenic type of disease[4-6].

By utilizing next-generation sequencing (NGS), many genetic disorders associated with epithelial defects or immunodeficiencies have been found in patients[7-9]. Notably, interleukin 10 receptor alpha subunit (IL10RA) dysfunction is the most common cause of the disease in East Asians, particularly in the Chinese, Japanese, and Korean populations[10-12].

According to our previous retrospective study, increased serum ferritin levels in VEO-IBD patients are indicative of monogenic disease, and very high serum levels of IL-10 suggest that patients with VEO-IBD are more likely to have *IL10RA* mutations [13].

We report four cases clinically diagnosed with VEO-Crohn's disease with high serum IL-10 levels, indicating *IL10RA* dysfunction. However, neither results of targeted gene panel sequencing (TGPS) nor those of whole-exome sequencing (WES) in the probands were conclusive. Whole-genome sequencing (WGS) was performed in two patients, and a novel 333-bp deletion in *IL10RA* was identified. The results of trio-WES for the other two patients were subsequently reanalyzed, and the same novel

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

333-bp deletion was found.

MATERIALS AND METHODS

Patients

Four patients with VEO-IBD, including two boys and two girls, were enrolled in our study. The medical history and clinical characteristics of the patients are summarized in Table 1. All patients were of Chinese Han ethnicity and were born to parents who were non-consanguineous, presented disease at the age of less than 1 year (range: 11 d to 8 mo), and experienced severe diarrhea with fistulas in the perianal region; blood samples were collected from three healthy volunteers.

Written informed consent was obtained from the parents of the four patients who participated in the study. This study was approved by the Institutional Review Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (No. 2019-15).

Whole-genome sequencing

Sample preparation and WGS were carried out by Beijing Berry Genomics Co., Ltd. (Beijing, China). The quality of the isolated genomic DNA was verified using the following two methods: (1) DNA degradation and contamination were monitored by electrophoresis on 1% agarose gels; and (2) DNA concentration was measured using the Qubit DNA Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States).

A total of 1 μg DNA per sample was used as the input material for DNA library preparation. The DNA sequencing library was generated using the CLEANNGS DNA kit following the manufacturer's recommendations, and indexing codes were added to each sample. Briefly, genomic DNA samples were enzymatically disrupted to a size of 350 bp. The DNA fragments were then end-polished, a-tailed, and ligated with a full-length adapter for Illumina sequencing, followed by further polymerase chain reaction (PCR) amplification. After the PCR products were purified (AMPure XP system, Beckman, Brea, CA, United States), libraries were analyzed to determine theirsize distribution using an Agilent 2100 Bioanalyzer (Santa Clara, CA, United States) and quantified by qPCR.

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the NovaSeq 5000/6000 S4 Reagent Kit (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on an Illumina NovaSeq 6000 platform, and 150-bp paired-end reads were generated.

The pathogenicity of all mutations was further evaluated according to the American College of Medical Genetics and Genomics guidelines.

Isolation and stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated according to a previous study, with minor modifications[14]. Briefly, blood was drawn from patient D and healthy controls by standard venipuncture in our pediatric ward and collected into a tube containing ethylenediamine tetraacetic acid. Blood (4 mL) was diluted 1:1 with sterile RPMI 1640 medium (Hyclone, Logan, UT, United States) at room temperature (RT) and carefully dropped into a 15-mL tube (Corning, Inc., Corning, NY, United States) containing 4 mL Ficoll-Paque Plus (GE Healthcare, Little Chalfont, United Kingdom). Notably, diluted blood was present on the surface of the Ficoll gradient. The 15-mL tube was centrifuged at 800 × g at RT for 20 min (brake off), after which the buffy coat was carefully aspirated and transferred to another sterile 15-mL tube. After washing the cells with 5 mL RPMI 1640 medium three times by centrifugation at 400 × g for 15 min at RT, most of the supernatant, as much as possible, was pipetted off.

Cells were aspirated with complete RPMI 1640 (10% fetal bovine serum and 1% penicillin-streptomycin) and cultured in 6-well plates at a density of 2×10^6 cells/well. Four groups of PBMCs from patients or healthy controls were established as follows: Unstimulated phosphate buffered saline (PBS), lipopolysaccharide (LPS) (100 ng/mL), LPS (100 ng/mL) + IL-10 (20 ng/mL), and IL-10 (20 ng/mL)[15]. Cells were cultured in the indicated milieu for 12 h at 37 °C. Proteins in PBS-, LPS-, and IL-10-stimulated PBMCs were collected in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors for western blot analysis. The supernatants of the PBS-, LPS-, and LPS + IL-10-stimulated PBMCs were collected to determine tumor necrosis factor-α (TNF-α) level.

Table 1 Clinical and laboratory characteristics of patients

	Patient A	Patient B	Patient C	Patient D	Normal range
Sex	Male	Female	Male	Female	
Consanguinity	-	-	-	-	
Disease onset	3 mo	9 mo	11 d	18 d	
Diarrhea (times/d)	1	8	20	6	
Bloody stool	+	-	+	+	
Weight (kg) SDS	7.65 (-1.475)	6.2 (-1.4)	8.2 (-1.05)	6.3 (-1.15)	
Height (cm) SDS	76 (-1.7)	65.2 (-3.65)	78 (0.25)	67 (1)	
Perianal disease	+	+	+	+	
Extragastro-intestinal manifestations	Fever, UTI, Sepsis	Recurrent otitis media	Fever, respiratory infetion	Fever, UTI, eczama	
WBC ($\times 10^9$)	12.4	19.44	15.34	24.5	3.69-9.16
Hemoglobin (g/L)	101	77	107	96	113-151
Platelet ($\times 10^9$)	624	621	424	387	101-320
IL-10 (pg/mL)	87.3	106	43.4	80.4	< 12.9
TNF-α (pg/mL)	20.7	30.5	3.9	19.8	< 16.5
ESR (mm/h)	6	55	19	23	F: 0-20; M: 0-15
Albumin (g/L)	28	29	33	23	35-55
Ferritin	17	47.3	46.5	68.2	11-306.8
Identified mutation	*IL10RA* (c.301C>T, p.R101W): Exon 1 del	*IL10RA* (c.537G>A, p.T179T): Exon 1 del	*IL10RA* (c.537G>A, p.T179T): Exon1 del	*IL10RA* (exon.1 del): Exon 1 del	

SDS: Standard deviation score; UTI: Urinary tract infection; TNF-α: Tumor necrosis factor α; IL10RA: Interleukin-10 receptor α subunit; del: Deletion; WBC: White blood cell.

Western blot analysis

Western blotting was performed as described previously[16]. Polyvinylidene fluoride membranes were blotted with monoclonal antibodies against phos-signal transducer and activator of transcription 3 (STAT3) (Tyr705), phos-STAT3 (Ser727), STAT3 (Cell Signaling Technology, Danvers, MA, United States), and glyceraldehyde-3-phosphate dehydrogenase (Servicebio, Wuhan, China). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Cell Signaling Technology) secondary antibodies were detected using a chemiluinescent substrate (Millipore, Billerica, MA, United States). Images were captured using an automatic chemiluminescence image analysis system (Tanon, Shanghai, China).

Enzyme-linked immunosorbent assay

The supernatant of PBMCs after stimulation with PBS, LPS or LPS + IL-10 was collected. IL-10 and TNF-α levels were determined using sandwich ELISA kits (DAKEWE, Shenzhen, China) according to the manufacturer's instructions.

Statistical analysis

Continuous variables are presented as the mean ± SEM, and the unpaired two-tailed Student's *t*-test or analysis of variance was used to compare the differences between groups as appropriate. Bonferroni correction was used for pairwise comparisons (GraphPad Prism v.5.0 software; GraphPad, Inc., La Jolla, CA, United States). Statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics of four patients with VEO-IBD

All four patients had severe diarrhea (> 6 times/d) and hematochezia during the first year of life. Patients C and D suffered from the disease during the newborn period. In addition to gastrointestinal symptoms, all cases exhibited extraintestinal manifestations, such as perianal abscesses, skin tags (Figure 1A), rectoperineal fistula, failure to thrive, recurrent otitis, urinary tract or respiratory infection, folliculitis, and even sepsis (Table 1). Elevated numbers of peripheral white blood cells and platelets and decreased hemoglobin and albumin levels were found in each patient. Remarkably, immune-related investigations showed that all patients had high serum levels of IL-10 (Table 1). All patients underwent colonoscopy and intestinal biopsy under general anesthesia, revealing erosive lesions (Figure 1B), and were diagnosed with Crohn's disease.

Identification of a novel compound heterozygous mutation in IL10RA

Before admission, all patients underwent TGPS or trio-WES. Two heterozygous pathogenic variants of *IL10RA* were detected in three patients (patient A: c.301C>T, p.R101W; patients B and C: c.537G>A, p.T179T) (Figure 1C and Supplementary Figures 1 and 2). Mutation of c.537G>A occurred at the exon-intron boundary of exon 4, which is a variant hotspot and disrupts RNA splicing (Figure 2A). No pathogenic or likely pathogenic variants were found in patient D.

As *IL10RA* mutation causes infantile IBD in an autosomal recessive manner and serum levels of IL-10 were very high in the four infantile patients with IBD, which is a valuable clinical indicator for identifying infantile IBD as a monogenic disease as we demonstrated previously[13], we suspected that mutations had been overlooked in WES owing to the techniques' limitations. After performing WGS in patients A and B, the breakpoints of the novel deletion were identified by manual review and correction. The deletion was located at chr11:117857030 upstream of exon 1 and chr11:117857362 in intron 1, which contains the 5'-untranslated region (UTR), all of exon 1, and part of intron 1 in *IL10RA* (Figure 1D and Figure 2B). PCR revealed a paternally-inherited 333-bp deletion in addition to the point mutations mentioned above (Figure 1E). The deletion and point mutations were inherited from both parents and eventually constituted compound heterozygotes in patients B (Figure 1F) and A (Supplementary Figure 1).

After identifying the 333-bp deletion in the gene in patients A and B, we reanalyzed the trio-WES data for patients C and D, specifically in the region from 117857030 and 117857362 on chromosome 11 and detected the same deletion. Patient C was a compound heterozygous carrier for c.537G>A, p.T179T (maternal), and the 333-bp deletion (paternal) (Supplementary Figure 2), and patient D was homozygous for the 333-bp deletion.

Histological and functional analysis of patient DIL10RA deletion

Histological findings in a specimen from the colon obtained during colonoscopy revealed oval-shaped intramural abscesses in the submucosa (Figure 3A). Figure 3B shows a higher magnification of inset 1 in Figure 3A, depicting intramural microabscesses.

To determine whether the novel 333-bp deletion in *IL10RA* caused IL-10R dysfunction and subsequently inhibited TNF-α production, supernatants of cultured PBMCs were collected and used to determine TNF-α levels. In healthy controls, LPS stimulation caused a remarkable increase in TNF-α production, whereas addition of IL-10 significantly decreased its abundance. Although LPS led to increased TNF-α production in patient D, this phenomenon was not reversed by the addition of IL-10, as observed in the healthy controls (Figure 3C).

To clarify the exact mechanisms involved, PBMCs were isolated from patient D because the patient was homozygous for the *IL10RA* deletion. PBMCs were stimulated with LPS in the presence or absence of IL-10. The results of western blot analysis showed that in PBMCs from healthy controls, both LPS and IL-10 stimulation caused an increase in the phosphorylation of STAT3 at Tyr705 but not at Ser727 (Figure 3D). In PBMCs from patient D, LPS stimulation also induced increased phosphorylation of STAT3 at Tyr705 but not at Ser727. However, IL-10 stimulation failed to significantly increase phosphorylation of STAT3 at Tyr705 in PBMCs of patient D compared with that in PBS-stimulated PBMCs. No significant differences in STAT3 phosphorylation at Ser727 were observed among the PBS-, LPS-, and IL-10-stimulated PBMCs from patient D (Figure 3D).

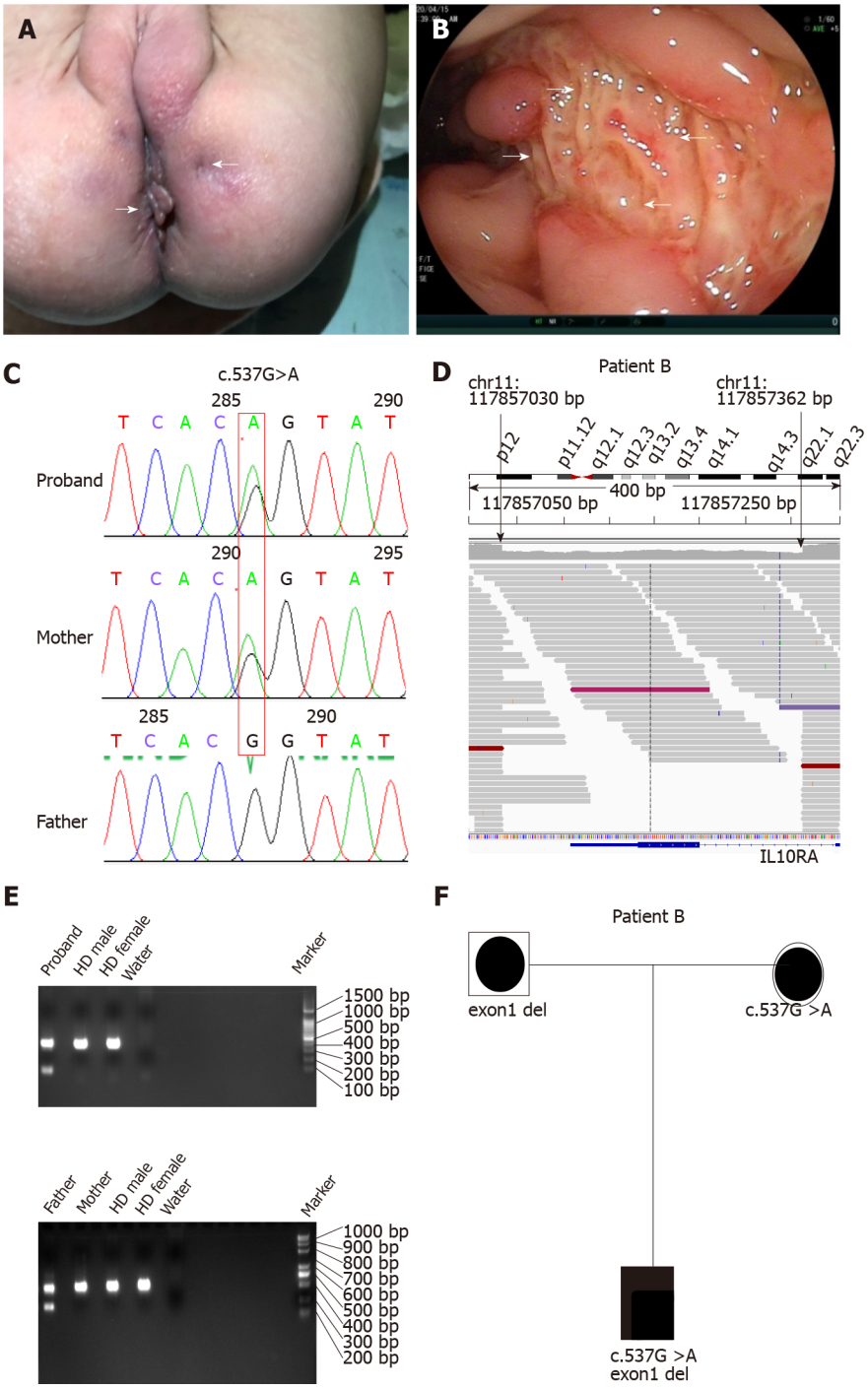

Figure 1 Identification of a novel 333-bp deletion spanning interleukin 10 receptor alpha subunit exon1. A: Perianal skin tag; B: Endoscopic image of ulcerations; C: Sanger DNA sequencing verified a compound heterozygous variant (c.537G>A) inherited from the mother in patient B; D: Whole-genome sequencing (WGS) data showing sequencing read pairs at breakpoints chr:117857030 and chr:117857362 of interleukin 10 receptor alpha subunit (*IL10RA*); E: Polymerase chain reaction validated the heterozygous deletion of 333 bp spanning exon1 inherited from the father; F: WGS revealed compound heterozygous variants of *IL10RA* in patient B with very early-onset inflammatory bowel disease. bp: Base pair; HD: Healthy donor.

DISCUSSION

VEO-IBD is challenging to diagnose and treat because the patients are critically ill and exhibit numerous potential mono-genic defects. Approximately 56 Mendelian genetic defects that can lead to IBD-like colitis have been identified, some of which show almost 100% penetrance, such as defects in *IL-10*, *IL10RA*, *IL10RB*, *FoxP3*, and *XIAP*[1]. NGS has led to breakthroughs in the diagnosis of genetic diseases, including monogenic VEO-IBD. According to a recent single-center study performed by Crowley *et al*[17], 7.8% of VEO-IBD (141 patients) and 13.8% of infantile-onset IBD (29 patients) cases had rare variations associated with monogenic genes. This prevalence was lower

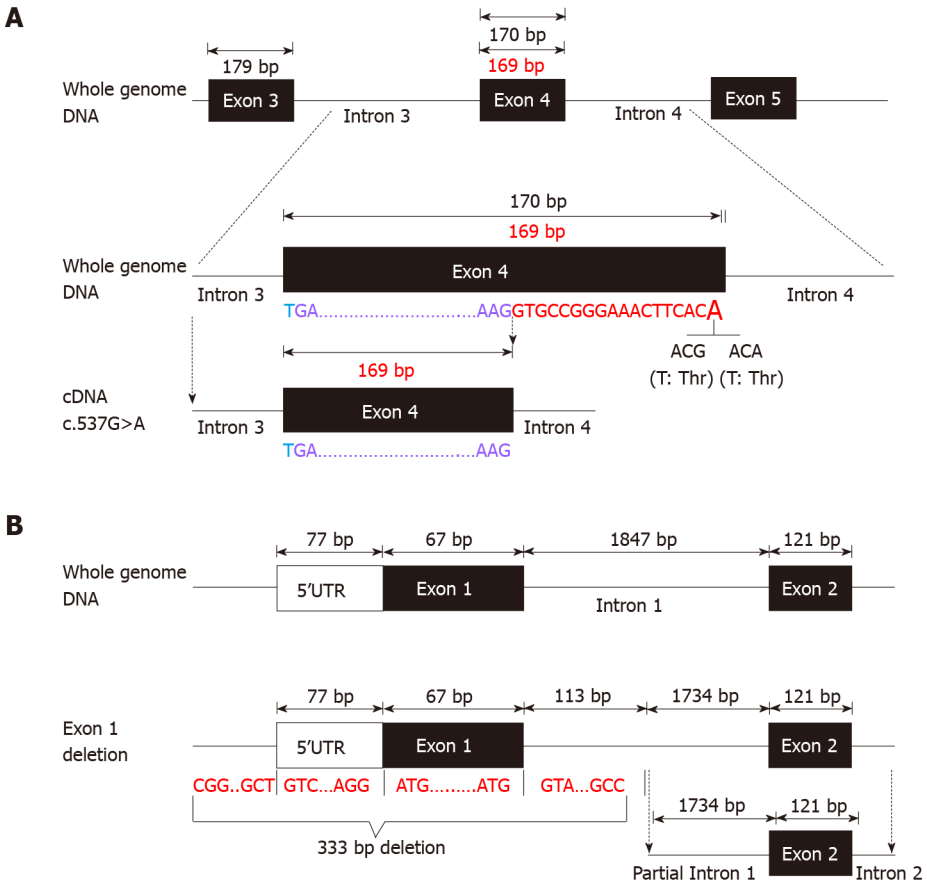

Figure 2 Schematic diagram of disrupted RNA splicing and 333-bp deletion in interleukin 10 receptor alpha subunit. A: Schematic diagram of disrupted splicing caused by c.537G>A in interleukin 10 receptor alpha subunit (*IL10RA)* between the boundary of exon4 and intron4; B: Schematic diagram of *IL10RA* 333-bp deletion. bp: Base pair.

than that reported in Chinese or European studies, which was approximately 31.9%-45.2%[12,18]. In our center, we found that 60.3% of patients with infant-onset IBD had monogenic disease, with mutations in *IL10RA* identified as the most common defect [13].

We evaluated four patients who had Crohn's disease from early infancy and exhibited failure to thrive, severe perianal disease, and resistance to medication. These characteristics indicate the presence of underlying genetic conditions[7]. Although the patients underwent NGS in a local hospital, neither TGPS nor WES revealed conclusive results. However, all patients showed very high serum IL-10 levels, and three patients had a disease-causing heterozygous mutation in *IL10RA*. According to our previous research, serum IL-10 levels > 33.05 pg/mL in patients with VEO-IBD strongly indicates the presence of IL10RA dysfunction[13]. Thus, we predicted that additional *IL10RA* mutations were missed during TGPS or WES. We did not detect the 333-bp deletion in *IL10RA* in two patients until WGS was performed. We then analyzed the same deletion in the other two patients by reanalyzing the trio-WES data. Finally, all patients were precisely diagnosed with VEO-IBD owing to compound heterozygous mutations in *IL10RA* in three patients and homozygous deletion involving *IL10RA* in one patient.

NGS, including TGPS and WES, is a powerful tool for identifying Mendelian genetic diseases in patients with VEO-IBD. The position paper on VEO-IBD by NASPGHAN/ESPGHAN suggests that NGS combined with the patient clinical history represents a vital component of the diagnostic approach[1]. A previous multicenter study showed that molecular diagnosis was achieved in 32% of patients with VEO-IBD when NGS was employed[18]. However, clinical NGS applications have limitations such as short read lengths, relatively high error rates, and incomplete coverage. Non-coding, yet potentially functional regions, and approximately 5% of exons are poorly covered in WES[19]. It is difficult to detect variants involving extensive deletions/insertions or short tandem repeats[20]. Charbit-Henrion *et al*[18] reported three WES-negative cases harboring large deletions in *LRBA* and *NCF1*. Compared to WES, WGS can detect all single-nucleotide variants, small indels, large

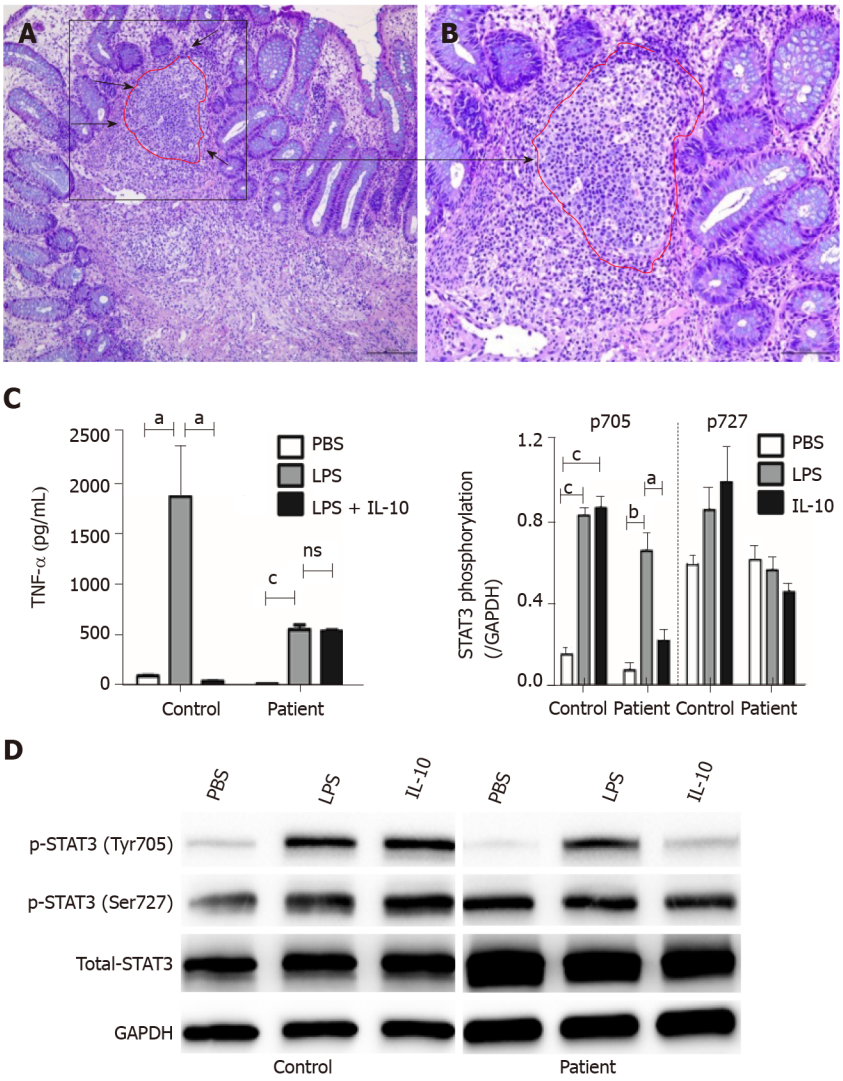

Figure 3 Histopathochemistry and functional results of homozygous interleukin 10 receptor alpha subunit mutation. A: Histological findings in a colonic specimen obtained during colonoscopy showing oval-shaped intramural abscesses; B: Higher magnification of inset A; C: Determination of TNF-α levels in the supernatant of cultured PBMCs from patient D after stimulation with PBS, LPS, or LPS + IL-10 *in vitro*; D: Western blot results of PBMCs isolated from patient D after stimulation with LPS or IL-10. Phosphorylation of STAT3 at Tyr705 and Ser727 and total STAT3 protein were detected (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ were considered as statistically different.

indels, and copy number variants. In our cases, the 333-bp *IL10RA* deletion contained a 5′-UTR, exon 1, and part of intron 1. This large deletion was easy to overlook when WES was used because of its technical limitations and insufficient bioinformatics analysis. After detecting this deletion, we requested re-analysis of the WES data for patients C and D, specifically for the 333-bp region spanning *IL10RA* exon 1. As expected, these two patients harbored the deletion. These results indicate that WES can detect the 333-bp deletion, which was easily overlooked in bioinformatics analysis because of algorithm defects and insufficient experience. WGS compensates for the limitations of WES. Our results indicated that WGS should be performed in VEO-IBD cases with negative WES results, particularly in those with infantile-onset IBD and treatment failure.

A comprehensive range of defects may be associated with VEO-IBD. It is difficult to differentiate every patient based on these defects and their underlying genetic disorders. A small number of patients show distinct phenotypes associated with specific functions, such as IL-10/IL-10R defects, IPEX, CGD, and XIAP[1]. *IL10RA* mutation-induced VEO-IBD shows specific characteristics such as refractory pan-colitis, perianal defects, fistulas, and growth failure, which occur during the neonatal period. An assay that can detect the lack of IL-10 inhibition in LPS can confirm receptor mutations[1]. Nevertheless, this assay is complicated and not routinely available in most hospitals in China. In a retrospective study, we found that the assay was useful for diagnosing IL10RA defects when the serum level of IL-10 was > 33.05

pg/mL. The assay sensitivity was very close to 100%, and the specificity was approximately 84%[13]. Elevated serum levels of IL-10 in patients with VEO-IBD indicated that even such high level of IL-10 could not inhibit TNF-α release and alleviate inflammation. Thus, serum IL-10 level may be a substitute for determining IL-10 inhibition in LPS functional testing. Therefore, when classic symptoms and laboratory results indicate that patients may have IL-10/IL-10R dysfunction but WES is inconclusive, *IL-10/IL10RA/IL-10RB* must be investigated and analyzed. It is recommended to use WGS or specific PCR to detect whether a large deletion involving these genes has occurred.

Interestingly, TNF-α production in LPS-stimulated PBMCs was not as robust in patient D as in control subjects; in our study, patient D was administered with anti-TNF-α antibody (Infliximab) before blood collection, which may have led to relatively low TNF-α production in the supernatant of LPS-stimulated PBMCs compared to that in healthy controls. Another possible reason is that increased TNF-α in the blood of patient D led to activation of the TNF-α receptor, resulting in JNK signaling-dependent inhibition of Bcl-2 expression, which acts as a major anti-apoptosis protein[21].The lack of a comprehensive functional test for *IL10RA* mutation is the major limitation of our study, although we conducted western blotting to determine the possible mechanism of *IL10RA* mutation-induced dysfunction of IL10RA signaling. Further studies are needed to explore other potential differences in IL10RA signaling.

CONCLUSION

Using WGS, we identified a novel 333-bp deletion in *IL10RA* that contributed to four cases of clinically diagnosed VEO-IBD with inconclusive *IL10RA* mutations. Most importantly, we confirmed that typical clinical manifestations and increased serum levels of IL-10 strongly indicate the existence of IL-10R dysfunction even when WES results are negative.

ARTICLE HIGHLIGHTS

Research background

Interleukin 10 receptor alpha (*IL10RA*) gene mutations constitute the most common monogenic disease in East Asia, affecting the health of children. However, identifying disease-causing mutant sites or copy number variants remains challenging in the clinic.

Research motivation

According to the results of our previous study, severe clinical symptoms as well as significantly increased serum IL-10 indicate IL10RA dysfunction, a monogenic phenotype of very early-onset inflammatory bowel disease (VEO-IBD). In addition, such very early-onset IBD showed a heterozygous *IL10RA* gene mutation by whole exon sequencing, leading to the employment of WGS and subsequent identification of 333-bp deletions in *IL10RA*.

Research objectives

We investigated the potential disease-causing gene mutations missed by WES and target gene panel sequencing (TGPS). Our results may contribute to monogenic disease diagnosis.

Research methods

Four patients clinically diagnosed with VEO-IBD during the past 5 years were recruited for this study. Based on their severe clinical phenotypes and the fact that before hospitalization, three patients harbored an *IL10RA* mutation (c.301C>T, p.R101W in one patient; c.537G>A, p.T179T in two patients), as detected by TGPS and trio-WES, and because WES did not show conclusive results in the fourth patient, we performed whole-genome sequencing (WGS) on patients A and B and reanalyzed the trio-WES data from patients C and D. To verify the functional change caused by the novel mutation, peripheral blood mononuclear cells (PBMCs) from patient D were isolated and stimulated *in vitro* with lipopolysaccharide (LPS), IL-10, and LPS + IL-10. Serum IL-10 levels in four patients and tumor necrosis factor-α (TNF-α) in the cell

supernatant were determined by ELISA. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Tyr705 and Ser727 in PBMCs was determined by western blot analysis.

Research results

Results of WGS revealed a novel 333-bp deletion encompassing exon 1 of *IL10RA* in patients A and B, which was also found in patients C and D after reanalyzing their WES data. Patient D was homozygous for the 333-bp deletion. All four patients showed elevated serum IL-10 levels. *In vitro*, IL-10-stimulated PBMCs from patient D failed to induce STAT3 phosphorylation at Tyr705 and minimally suppressed TNF-α production induced by LPS. Phosphorylation at Ser727 in PBMCs was not affected by LPS or LPS + IL-10 in both healthy subjects and patient D.

Research conclusions

Genome-wide uniformity of coverage of WGS identified a novel 333-bp deletion in *IL10RA* in four patients with VEO-IBD, whereas the results of initially performed WES were inconclusive. WGS, which was more informative than WES, is the most important comprehensive second-tier genomic test for monogenic diseases in the clinic.

Research perspectives

We will customize a multiplex ligation-dependent amplification probe of the 333-bp deletion in *IL10RA* to help diagnose *IL10RA* mutation-related monogenic diseases.

ACKNOWLEDGEMENTS

The authors would like to thank the members of the Department of Pediatrics and Pediatric Laboratory.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 November 28; 27(44): 7716-7733

DOI: 10.3748/wjg.v27.i44.7716

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

SYSTEMATIC REVIEWS

Proton pump inhibitors and colorectal cancer: A systematic review

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Conflict-of-interest statement: All the authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 checklist, and the manuscript was prepared in accordance with the PRISMA guidelines.

Country/Territory of origin: Poland

Specialty type: Gastroenterology and hepatology

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Abstract

BACKGROUND

The use of proton pump inhibitors (PPI) is common worldwide, with reports suggesting that they may be overused. Several studies have found that PPI may affect colorectal cancer (CRC) risk.

AIM

To summarize current knowledge on the relationship between PPI and CRC from basic research, epidemiological and clinical studies.

METHODS

This systematic review was based on the patients, interventions, comparisons, outcome models and performed according to PRISMA guidelines. MEDLINE, EMBASE, Scopus, and Web of Science databases were searched from inception until May 17, 2021. The initial search returned 2591 articles, of which, 28 studies met the inclusion criteria for this review. The studies were categorized as basic research studies (n = 12), epidemiological studies (n = 11), and CRC treatment studies (n = 5). The quality of the included studies was assessed using the Newcastle-Ottawa Scale or Cochrane Risk of Bias 2.0 tool depending on the study design.

RESULTS

Data from basic research indicates that PPI do not stimulate CRC development *via* the trophic effect of gastrin but instead may paradoxically inhibit it. These studies also suggest that PPI may have properties beneficial for CRC treatment. PPI appear to have anti-tumor properties (omeprazole, pantoprazole), and are potential T lymphokine-activated killer cell-originated protein kinase inhibitors

Unsolicited article; Externally peer reviewed.

Peer-review report's scientific quality classification

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

Received: July 4, 2021
Peer-review started: July 4, 2021
First decision: July 13, 2021
Revised: July 14, 2021
Accepted: September 8, 2021
Article in press: September 8, 2021
Published online: November 28, 2021

P-Reviewer: Yoshimatsu K, Zhang Y
S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Li JH

(pantoprazole, ilaprazole), and chemosensitizing agents (pantoprazole). However, these mechanisms have not been confirmed in human trials. Current epidemiological studies suggest that there is no causal association between PPI use and increased CRC risk. Treatment studies show that concomitant PPI and capecitabine use may reduce the efficacy of chemotherapy resulting in poorer oncological outcomes, while also suggesting that pantoprazole may have a chemosensitizing effect with the fluorouracil, leucovorin, oxaliplatin (FOLFOX) regimen.

CONCLUSION

An unexpected inhibitory effect of PPI on CRC carcinogenesis by way of several potential mechanisms is noted. This review identifies that different PPI agents may have differential effects on CRC treatment, with practical implications. Prospective studies are warranted to delineate this relationship and assess the role of individual PPI agents.

Key Words: Colorectal cancer; Proton pump inhibitor; Carcinogenesis; Cancer epidemiology; Capecitabine; Translational medicine

Core Tip: Proton pump inhibitors (PPI) are a widely, often inappropriately, used class of drugs. Through various mechanisms, they are suspected to increase the risk of gastrointestinal cancers, including colorectal cancer (CRC). The aim of this review is to summarize existing literature on the effect of PPI on CRC. The review assessed basic research studies to identify mechanisms at play in this relationship, observational studies to determine if a causal association exists between PPI use and CRC incidence, and clinical studies to examine if PPI use during chemotherapy influences treatment efficacy and oncological outcomes.

Citation: Patel A, Spychalski P, Antoszewska M, Regula J, Kobiela J. Proton pump inhibitors and colorectal cancer: A systematic review. *World J Gastroenterol* 2021; 27(44): 7716-7733
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7716.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7716

INTRODUCTION

Proton pump inhibitors (PPI) are among the most widely prescribed medications globally[1,2]. Since their development in the 1980s, these drugs have been used for conditions such as peptic ulcer disease, gastroesophageal reflux disease, stress gastritis, and gastrinomas[3]. PPI are available by prescription, but are also sold over-the-counter resulting in frequent use without appropriate indication[4,5]. The mechanism of action of PPI involves irreversible, long-lasting binding to and inhibition of the hydrogen-potassium adenosine triphosphatase (ATPase) enzyme system on gastric parietal cells[6]. These ATPase pumps are responsible for secreting H^+ ions into the gastric lumen, resulting in the production of gastric acid. Suppression of gastric acid production by PPI lowers the acidity of gastric contents while causing feedback hypergastrinemia.

Gastrin, in turn, is a potent growth factor involved in several physiological and pathological processes, including neoplastic transformation[7]. One hypothesis suggests that gastrin may have pro-inflammatory properties and can stimulate the tumor microenvironment *via* macrophage activation and chemotaxis. It is therefore possible that PPI and the resultant hypergastrinemia have a cancer-promoting effect [8].

Some studies, however, suggest that PPI may also exert anti-tumor properties. These drugs might paradoxically inhibit the proliferative effects of hypergastrinemia while demonstrating anti-oxidant, anti-inflammatory, and pro-apoptotic activity[9]. PPI could also have a potential chemotherapeutic role by reducing tumor resistance to chemotherapeutics. De Milito *et al*[10] reported that manipulating cancer pH may

sensitize them to certain chemotherapeutics. In contrast, the TRIO-013/LOGiC trial demonstrated that PPI may negatively affect the efficacy of some cytotoxic drugs, possibly due to alkalinization of the gastric environment[11].

Overall, concerns are increasing regarding the safety of PPI use because of induced hypergastrinemia and a possible association with gastrointestinal (GI) cancer risk, including colorectal cancer (CRC). Many current patients with CRC may have a history of PPI use, but precise epidemiological data are not available. Ahn *et al*[12] and Ma *et al*[13] have summarized observational studies assessing the association of PPI use with the risk of developing CRC. Ahn *et al*[12] found no significant effect of PPI on CRC risk, whereas Ma *et al*[13] found a weak association between long-term PPI use (> 5 years) and increased CRC risk. However, there are no systematic reviews summarizing the evidence from basic research studies exploring mechanisms by which PPIs may affect CRC and from human epidemiological and clinical studies examining PPI use in the context of CRC survival and treatment. As a systematic review might identify important epidemiological and clinical findings, our aim was to provide a comprehensive report on the association of PPI use and CRC based on recent basic research and human studies.

MATERIALS AND METHODS

Literature search

This systematic review was performed in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement using PICO (patients, interventions, comparisons, outcomes)-based questions. Following a predefined search strategy, we searched the MEDLINE, EMBASE, Web of Science, and Scopus online databases to identify suitable articles. No filters were applied during the search, and we also performed backward citation chaining of eligible full-text studies.

Evidence acquisition

On May 17, 2021, two independent researchers (AP, PS) performed a search of the target online databases for eligible studies. The search string was ("*proton pump inhibitors*" or "*proton pump inhibitor*" or "*ppis*" or "*ppi*" or "*omeprazole*" or "*pantoprazole*" or "*esomeprazole*") and ("*CRC*" or "*colorectal cancer*" or "*colon cancer*" or "*rectal cancer*"). The preliminary search returned 2591 articles, which two independent researchers (AP, PS) screened. The entire protocol is presented in a PRISMA flowchart (Supplementary Figure 1).

Inclusion and exclusion criteria

We used PICO framework-based research questions for this review (Supplementary Table 1). If articles met predefined criteria, they were included and categorized as basic research (animal and cell studies), epidemiological (incidence and mortality studies), and treatment studies. Articles were excluded if the full text was not available or was not in English, were not original articles, or did not conform with PICO.

Evidence synthesis and Quality Assessment:

Two independent researchers (Patel A and Spychalski P) retrieved and summarized information from the eligible studies in tables. The authors (Patel A, Spychalski P, Antoszewska M and Kobiela J) discussed conflicts regarding inclusion of studies and resolved them by consensus. Two independent researchers (Patel A and Antoszewska M) assessed the quality of included case-control and cohort studies using the Newcastle-Ottawa scale (NOS)[14]. This scale awards a maximum of nine points for each of the following items: Selection (four stars), comparability (two stars) and outcomes (three stars). Studies were considered of high quality if they scored seven or more stars on NOS assessment. Additionally, the Cochrane Risk of Bias 2.0 tool was used to assess bias in randomized controlled studies included in the retrospective post-hoc analysis reports[15]. The results of quality assessment are described in Supplementary Material along with Supplementary Table 2.

RESULTS

A total of 28 studies were included in the review: Basic research studies (n = 12) [animal models (n = 5), CRC cell lines (n = 1), or both (n = 6)]; epidemiological studies (

n = 11) [analyzing CRC risk (n = 9) and survival (n = 2) associated with PPI use]; and treatment studies (n = 5), examining the effects of PPI on CRC chemotherapy regimens.

Basic studies

The included basic studies examined two primary themes: (1) Trophic effects of PPI-induced hypergastrinemia; and (2) Potential chemotherapeutic role of PPI as cytostatic drugs, chemosensitizing drugs, or T lymphokine-activated killer cell-originated protein kinase (TOPK) inhibitors. The information from the basic studies is summarized in Tables 1 (animal models) and 2 (CRC cell lines).

Trophic studies: The trophic effects of PPI-induced hypergastrinemia were investigated in six studies[16-21]. Four animal studies demonstrated that PPI-induced hypergastrinemia did not influence growth and invasiveness of CRC[16-19]. These studies showed that omeprazole treatment resulted in significantly higher serum or plasma gastrin levels (4- to 20-fold across studies) in comparison to control groups. However, the treated and control groups were similar in terms of tumor burden and/or invasiveness of CRC. Graffner *et al*[16] found omeprazole-treated and control mice to be similar in terms of tumor size, survival and distant metastasis rate. Pinson *et al*[17] compared low-dose and high-dose omeprazole, ranitidine (histamine-2 receptor antagonist), and control exposure in rats. They found that overall tumor burden and survival were similar among these groups but documented significantly lower mean tumor number, volume, and total mass in the ranitidine group (multiple comparisons, all $P < 0.05$). Hurwitz *et al*[18] reported concordant findings in their study, additionally noting no significant differences in DNA, RNA or protein concentration in tumor-free colonic tissues of treated *versus* control rats. Chen *et al*[19] performed sham operation, colostomy and/or fundectomy, omeprazole treatment, or fasting with refeeding to assess the short-term and long-term effects of hypergastrinemia. None of the groups demonstrated growth of CRC tumors, but the fundectomy group showed suppressed tumor growth.

Two studies indicated that PPI treatment resulted in suppression of CRC growth[20, 21]. Penman *et al*[20] found a significantly lower incidence of CRC tumors in omeprazole-treated rats than controls (63% *vs* 95%, $P < 0.02$). They hypothesized that omeprazole possibly influenced metabolism of the carcinogen (azoxymethane) by affecting either intestinal microflora or P450 isoenzymes, therefore resulting in lower CRC growth. Working with the NCI-H719 human colon cancer cell line, Tobi *et al*[21] demonstrated a dose-dependent decrease in proliferation (cytostatic effect) with omeprazole, but noted no such effect in two other cell lines (DLD-1 and LCC-18). These researchers found that the cytostatic effect of omeprazole persisted even when omeprazole was combined with gastrin, suggesting a potential paradoxical inhibition of gastrin's trophic influence on CRC.

Chemotherapeutic studies: Six studies addressed the potential chemotherapeutic role of PPI in CRC[22-27]. Three studies assessed the cytotoxic effects – anti-proliferative, pro-apoptotic, and anti-inflammatory properties – of PPI on CRC and found that PPI (omeprazole, pantoprazole) dose-dependently inhibited proliferation and induced apoptosis in CRC models[22-24]. Patlolla *et al*[22] reported that omeprazole resulted in upregulation of p21waf1/cip1 and downregulation of cyclin A, Bcl-2, Bcl Xl, and survivin expression, leading to induction of cell apoptosis. Kim *et al*[23] reported on the anti-inflammatory activities of PPI, describing reduced tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), colon thiobarbituric acid-reactive substance (TBA-RS), and expression of cyclooxygenase-2 (COX-2) and NO synthetase. These authors also suggested a potential anti-proteolytic and anti-mutagenic action of PPI, reporting decreased levels of matrix metalloproteinase (MMP)-9, MMP-11, and MT1-MMP and decreased beta-catenin accumulation in omeprazole-treated mice as compared to controls.

Han *et al*[24] reported similar findings on the pro-apoptotic, anti-inflammatory, and anti-proliferative properties of PPI, along with a potential anti-angiogenic effect. They found that PPI treatment reduced expression of angiogenic factors such as interleukin (IL)-8, platelet-derived growth factor, vascular endothelial growth factor, and hypoxia-inducible factor 1-alpha. Moreover, Kim *et al*[23] and Han *et al*[24] demonstrated that PPI may paradoxically inhibit the trophic effect of gastrin on CRC cells. Kim *et al*[23] found cell proliferation to be significantly ($P < 0.05$) reduced in cells treated with both omeprazole and gastrin compared to with gastrin only. Han *et al*[24] reported similar findings and found that PPI antagonized gastrin's binding to cholecystokinin B receptor (CCKBR), both alone and in combination with gastrin.

Table 1 Summary of basic research studies (animal models)

Author	Aim of study	PPI investigated	Species strain, gender	Methods of CRC induction	PPI treatment	Experimental/ control group	Outcome measure	Main findings	Mechanism studied	Role of PPI in CRC
Graffner *et al*[16] 1992	To determine the influence of PPI-induced endogenous hypergastrinemia on growth in CRC-implanted mice	OME	BALB/C mice, M	MC-26 tumor cells injected SC in epigastric region	Daily for 19 d, 400 μmol/kg, PO	18/18	Tumor size, survival	5-fold higher serum gastrin levels in OME-treated animals than controls. No differences in tumor size, tumor weight, survival and metastatic potential (61% *vs* 72%, P = NR) between tumor-bearing treated and control group	Trophic effect of gastrin	NE
Penman *et al*[20] 1993	To assess the influence of OME-induced hypergastrinemia on CRC development in animal models	OME	Sprague-Dawley rats, F	12 (weekly) SC azoxymethane (10 mg/kg/wk)	Daily for 27 wk, 40 μmol /kg, PO	19/20	Number of tumors, position, volume; metastatic disease	9-10-fold higher gastrin levels in OME-treated groups than control groups. Significantly fewer OME-treated rats developed tumors compared to control group (63% *vs* 95%, $P < 0.02$). Number of tumors were also significantly lower in OME-treated rats. Average tumor size and invasiveness of CRC was similar for both groups	Trophic effect of gastrin	PE
Hurwitz *et al*[18] 1995	To evaluate effect of omeprazole-induced hypergastrinemia on carcinogen-induced CRC in rats	OME	Sprague-Dawley rats, M	Six (weekly) IP methylazoxymethanol (30 mg/kg)	Daily for 10 wk, 40 mg/kg, gastric gavage	NR	Number of tumors, volume and total tumor burden, biochemical and histological analysis	Serum gastrin levels were elevated 6-fold in OME-treated animals *vs* controls. No differences in tumor number, tumor volume, and total tumor burden between treated and control group. No histological (crypt/mucosal height) or biochemical features in CRC-free regions of colon	Trophic effect of gastrin	NE
Pinson *et al*[17] 1995	To assess if hypergastrinemia enhances progression or invasiveness of CRC	OME	Sprague-Dawley rats, M	Six (weekly) IP methylazoxymethanol (30 mg/kg)	Daily for 10 wk, 14 or 40 mg/kg, gastric gavage	162/108	Number of tumors, volume and total tumor burden, histological analysis	Plasma gastrin levels in the treated groups (low-dose OME, high-dose OME, ranitidine) were 3–5-fold higher than controls. Crypt height/mucosal height ratio of CRC-free colonic mucosa was similar between all groups. No significant differences in tumor number, tumor burden and invasiveness between OME-treated and control groups.	Trophic effect of gastrin	NE
Chen *et al*[19] 1998	To examine trophic effects of endogenous hypergastrinemia colonic mucosa and transplanted colon adenocarcinoma in rats	OME	Sprague-Dawley rats, M	Injection of K-12 cell line (Established in syngeneic BDIX rats *via* induction using 1,2-dimethylhydrazine)	Daily for 10 d, 400 μmol/kg, PO	NR	Tumor weight and volume, histological analysis, labelling index	OME treatment and fundectomy raised serum gastrin levels by 4-5-fold. OME-treatment did not stimulate growth of transplanted tumor (K-12) cells, while fundectomy suppressed CRC growth (decreased labelling index, weight and volume of tumor) Sustained hypergastrinemia did not affect the thickness and labelling index of normal colon mucosa	Trophic effect of gastrin	NE
Kim *et al* [23] 2010	To evaluate chemo-preventive properties of	OME	C57BL/6 mice, F	Colitis induction - 15 cycles of 0.7% DSS in drinking	NR, 10 mg/kg, IP	12/24	Tumor burden, biochemical and	OME-treated group developed significantly lower number of colon tumors than control	Cytostatic properties	PE

	omeprazole in a colitis-associated CRC mouse model			water			histological analysis	groups. OME administration also resulted in decreased inflammatory markers (TNF-α, serum NO, and colon TBA-RS levels), attenuated expression of MMP, COX-2, NO synthase, and β-catenin, and greater apoptotic index		
Patlolla *et al*[22] 2012	To assess chemo-preventive effects of omeprazole	OME	F344 rats, M	Two (weekly) SC azoxymethane (15 mg/kg)	9 wk, 200/400 ppm, PO	30/18	Aberrant crypt foci incidence	Omeprazole inhibited the AOM-induced colonic foci formation in a dose-dependent manner	Cytostatic properties	PE
Han *et al* [24] 2014	To study the effects of PPI on colitis-associated carcinogenesis	PAN	APCMin/+ mice, M	Genetically engineered mutation in APC gene	Thrice weekly for 10 wk, 8 mg/kg, IP	NR/8	Number and size of intestinal polyps	Gastrin + PPI exerted significant anti-polyposis effect through β-catenin inactivation, increased apoptosis, anti-angiogenic, and MAPK inactivation relevant to decreased levels of pro-inflammatory mediators	Cytostatic properties	PE
Zeng *et al*[26] 2016	To evaluate the effect of pantoprazole as TOPK inhibitor *in vivo* and *in vitro*	PAN	Non-obese diabetic-SCID mice	HCT 116 cells inoculated SC into left flank	Every 2 d for 19 d, 100 mg/kg, IP	8/8	Tumor volume, immunohistochemical analysis	Tumors treated with PAN grew significantly more slowly, and the size of tumors was smaller compared with the control group. PAN-treated group had lower average tumor volume per mouse compared to controls (111 mm^3 *vs* 285 mm^3, $P < 0.05$). Average body weight was similar throughout the study indicating no toxic effects of PAN in the mice IMHC for phosphorylated histone H3 revealed substantially decreased expression in PAN-treated group compared to control	TOPK inhibition	PE
Zheng *et al*[27] 2017	To evaluate the effect of PPI as a TOPK inhibitor *in vivo* and *in vitro*	ILA	CB-17/Icr-scid mice	HCT 116 cells inoculated SC into left flank	Daily for 19 d, 150 mg/kg, PO	8/8	Tumor volume, immunohistochemical analysis	Estimated tumor volumes of treatment groups were less than that of the control group. No toxicity or differences in body weight were observed. Expression levels of phosphorylated histone H3 were substantially decreased in ilaprazole-treated groups compared with the control group	TOPK inhibition	PE
Wang *et al*[25] 2017	To investigate the chemosensitizing potential of PPI in CRC	PAN	BALB/C mine, F	HT29 cells injected SC	Weekly for 4 wk, 30 mg/kg, IP	NR	Tumor burden	PAN combined with 5-FU demonstrated greater inhibition of tumor growth and smaller tumor sizes compared to 5-FU alone	Chemosensitizing properties	PE

AOM: Azoxymethane; CRC: Colorectal cancer; COX-2: Cyclooxygenase-2; F: Female; 5-FU: 5-Fluorouracil; ILA: Ilaprazole; IMHC: Immunohistochemistry; IP: Intraperitoneal; M: Male; MAPK: Mitogen-activated protein kinase; MMP: Matrix metalloproteinase; NO: Nitric oxide; NE: No effect; NR: Not reported; OME: Omeprazole; PAN: Pantoprazole; PO: Per os; PPI: Proton pump inhibitors; PE: Protective effect; SC: Subcutaneous; TOPK: T lymphokine-activated killer cell-originated protein kinase; TNF-α: Tumor necrosis factor-alpha; TBA-RS: Thiobarbituric acid-reactive substance.

Wang *et al*[25] found that PPI increased the chemosensitivity of human colon cancer cells (HT29 and RKO lines) as PPI combined with 5-fluorouracil (5-FU) resulted in significantly higher cell inhibition rates than 5-FU alone (*in vitro* experiment: $P = 0.04$; *in vivo* experiment: $P = 0.03$).

Table 2 Summary of basic research studies (colorectal cancer cell lines)

Author	Aim of study	PPI investigated	Cell lines studied	Outcome measure	Main finding	Mechanisms	Role of PPI in CRC
Tobi *et al*[21] 1995	To assess the direct effects of gastrin and OME on growth of CRC origin cells separately and in combination	OME	NCI-H716, LCC-18, DLD-1	Proliferation of cell lines	OME treatment resulted in cytostatic effect on 1 of the 3 cell (NCI-H716) lines tested. Dose-dependent decrease in cell proliferation noted compared to controls ($P < 0.05$). Effect seen with gastrin (low concentration), OME, or both in combination. Gastrin increased proliferation of NCI-H716 cells only at high concentrations	Trophic effect of gastrin	PE
Kim *et al* [23] 2010	To evaluate chemo-preventive properties of omeprazole in a colitis-associated CRC mouse model	OME	HT29	Cell viability and growth	Significant cleavage of capsase-3 in presence of 500 μmol/L omeprazole, but effect attenuated with gastrin pre-treatment, signifying that gastrin could attenuate the cytotoxicity of PPI by decreasing apoptosis. Compared with the gastrin-treated group, cell proliferation was significantly attenuated in the presence of omeprazole ($P < 0.05$), suggesting that PPI could offset the trophic action of gastrin on colon cells	Cytostatic properties	PE
Patlolla *et al*[22] 2012	To assess chemo-preventive effects of OME	OME	HCA-7, HCT-116	Cell viability, cytotoxicity assays, apoptotic assays	Dose-dependent suppression of cell growth and induction of apoptosis seen in both cell lines	Cytostatic properties	PE
Han *et al* [24] 2014	To study the effects of PPI on colitis-associated carcinogenesis	PAN	HCT116	Proliferation rate, apoptosis, and molecular analysis	PPI antagonizes trophic actions of gastrin, causes dose-dependent suppression of cellular viability. Combination of PPI and gastrin had higher cytotoxic activity than PPI alone. PPI alone or in combination with gastrin induces apoptosis and blocks gastrin-CCKBR binding. PPI may possess anti-angiogenic activity, which inhibits the expression of angiogenic factors induced by gastrin	Cytostatic properties	PE
Zeng *et al*[26] 2016	To evaluate the effect of pantoprazole as TOPK inhibitor *in vivo* and *in vitro*	PAN	HCT116, SW480, WiDr	Cell viability, TOPK assay analysis, cytotoxicity assays	Pantoprazole had different cytotoxicity toward different colon cancer cells. It inhibits anchorage-independent growth of colon cancer cells. Cell line with high TOPK activity (HCT116) was more sensitive to pantoprazole. The study suggests that TOPK is a direct target for pantoprazole to suppress colon cancer cell growth	TOPK inhibition	PE
Zheng *et al*[27] 2017	To evaluate the effect of PPI as TOPK inhibitor *in vivo* and *in vitro*	ILA	HCT116	Cell viability, TOPK assay analysis, cytotoxicity assays	Ilaprazole exhibited potent inhibitory effect on growth and induced apoptosis in HCT116 cells in a dose-dependent manner. The study suggests that TOPK was a direct target for ilaprazole to suppress cancer cell growth and its anticancer activities were dependent on the TOPK expression. Inhibition of TOPK by ilaprazole is dependent on TOPK abundance in cancer cells	TOPK inhibition	PE
Wang *et al*[25] 2017	To investigate the chemosensitizing potential of PPI in CRC	PAN	HT29, RKO	Cell inhibition rate	PPI in combination with 5-FU had a higher inhibitory effect on CRC cell line growth compared to controls. The study suggests that PPI may increase sensitivity of CRC tumors to 5-FU *in vitro*	Chemosensitizing properties	PE

CCKBR: Cholecystokinin-B receptor; CRC: Colorectal cancer; ILA: Ilaprazole; OME: Omeprazole; PAN: Pantoprazole; PPI: Proton pump inhibitors; PE: Protective effect; TOPK: T lymphokine–activated killer cell–originated protein kinase.

Zeng *et al*[26] and Zheng *et al*[27] investigated pantoprazole and ilaprazole, respectively, as potential TOPK inhibitors. Both groups found that PPI inhibited CRC cell growth *via* TOPK inhibition *in vitro* and *in vivo*. Among the PPI, ilaprazole and pantoprazole showed the strongest affinity for TOPK. Zeng *et al*[26] examining three colon cancer cell lines with different TOPK expression levels reported that pantoprazole had a growth-inhibiting effect through interaction with TOPK. Zheng *et al*[27] described similar results for ilaprazole, with PPI treatment resulting in decreased phosphorylation of histone, a TOPK-mediated process, suggesting that TOPK may be a direct target for these drugs. Furthermore, the authors found ilaprazole to be an inducer of apoptosis *via* activation of caspases and cleavage of poly-(ADP-ribose) polymerase.

Epidemiological studies

Six case-control studies[28-33], two prospective studies[34,35], and one retrospective study[36] addressed the incidence of CRC in PPI-users *versus* non-users. Two retrospective cohort studies assessed the survival of CRC patients in relation to PPI use.

Incidence studies: The information from the six included case-control incidence studies is abstracted in Table 3[28-33]. The time definition of PPI use varied across studies. The included studies analyzed information from healthcare databases or registries of different regions – Denmark, the Netherlands, United Kingdom, San Francisco (United States), and Washington (United States). A total of 31829 CRC patients matched with 276647 controls were included in this review. After adjustment for confounders, none of the studies revealed an increased risk of CRC in current or ever PPI-users in comparison to non-users. Furthermore, most (5/6) of the studies found that the duration of PPI use or average daily dose of PPI did not influence CRC risk[28-30,32,33]. However, Lee *et al*[31] reported that the risk of CRC increased significantly with ≥ 10 years of PPI use compared to no use [odds ratio (OR) = 1.28, 95% confidence interval (CI): 1.15-1.44]. Robertson *et al*[28], Yang *et al*[30] and Kuiper *et al*[33] did not find any significant increase in risk in recent or former PPI-users. However, Kuiper *et al*[33] found that current PPI-users were at an increased risk of developing CRC (OR = 1.30, 95%CI: 1.16-1.47), especially with concomitant non-steroidal anti-inflammatory drugs use (OR=1.57, 95%CI: 1.27-1.93).

Three cohort studies assessed the hazard of developing CRC in PPI-users and non-users[34-36] (Table 4). The review included a total of 108107 PPI-users and 609800 non-users identified through healthcare databases in Korea, United States, and Taiwan. Hwang *et al*[34] and Babic *et al*[35] found no significant association between PPI exposure and CRC development, but Lei *et al*[36] reported a significantly increased risk of CRC among PPI-users [hazard ratio (HR) = 2.03, 95%CI: 1.56-2.63, $P < 0.05$]. Hwang *et al*[34] reported that PPI use was associated with increased CRC risk in individuals at low risk for CRC (non-obese, non-diabetics, female, aged < 50 years, no history of alcoholism, receiving ≥ 180 daily defined dose of PPI) (HR = 12.30, 95%CI: 1.71-88.23, $P < 0.01$). Babic *et al*[35] found that the period of PPI use had no effect on CRC risk but that current PPI use was associated with a decreased risk (HR = 0.82, 95%CI: 0.68-0.98). In contrast, Lei *et al*[36] reported a time-dependent and dose-dependent relationship between PPI use and CRC development, with patients at higher risk if they were using PPI for ≥ 1 year and increasing doses of PPI. On further analysis, Lei *et al*[36] found that the risk of CRC was increased with esomeprazole, lansoprazole, and omeprazole, but no such association was seen with pantoprazole and rabeprazole.

Survival studies: Survival of CRC patients was assessed in two retrospective studies. Graham *et al*[37] included 1304 CRC (117 PPI-users at diagnosis) patients with similar baseline characteristics, but greater cardiac comorbidities in PPI-users ($P < 0.05$). The authors found similar overall survival (OS) rates at 1-, 2- and 5-years between PPI-users and non-users, but the cumulative survival of PPI-users was significantly shorter than non-users (1775 *vs* 2279 d, $P = 0.048$). Furthermore, after controlling for known risk factors, the risk of mortality was significantly higher in CRC patients using PPI (HR = 1.34, 95%CI: 1.01-1.78, $P = 0.04$). Tvingsholm *et al*[38] analyzed cancer-specific mortality for nine cancers in a cohort of 347919 patients, including 47188 CRC patients. They found that the risk of mortality in CRC patients was approximately 12 times higher in PPI-users as compared to non-users (HR = 11.8, 95%CI: 11.3-12.4).

Treatment studies

The effects of PPI use concurrently with chemotherapeutic treatment of CRC was

Table 3 Summary of epidemiological studies assessing the exposure of proton pump inhibitors in colorectal cancer patients

Author, year, place	Accrual year	Study design	Grouping	Number	Exposed	Unexposed	OR (95%CI)	Adjustments	Risk of CRC
Robertson *et al*[28] 2007 (Denmark)	1989-2005	CC	CRC patients	5589	295	5294	1.11 (0.97-1.27)	Age, sex, place of residence (matched), H2 blocker use, aspirin/NSAIDs, statins/diabetics use, history of cholecystectomy, alcohol	No increased risk
			Non-CRC control	55890	2692	53198			
Van Soest *et al*[29] 2008 (Netherland)	1996-2005	CC	CRC patients	594	53	541	0.85 (0.63-1.16)	Age, sex, calendar time, follow-up duration (matched), comorbidities	No increased risk
			Non-CRC control	7790	725	7065			
Yang *et al*[30] 2007 (United Kingdom)	1987-2002	CC	CRC patients	4432	769	3663	1.2 (0.8-1.9)	Age, sex, alcohol, smoking, BMI, H2 blocker use, aspirin/NSAID use, calendar time, follow-up, general practice site (matched), HRT use, history of colonoscopy/flexible sigmoidoscopy	No increased risk
			Non-CRC control	44292	5133	39159			
Lee *et al*[31] 2020 (San Francisco, United States)	1996-2016	CC	CRC patients	18595	1406	17189	NR	Age, sex, ethnicity, general practitioner site, enrolment duration, smoking, alcoholism, BMI, history of colonoscopy, family history of CRC, Crohn's disease, Ulcerative colitis	No increased risk
			Non-CRC control	160122	10813	149309			
Chubak *et al*[32] 2009 (Washington State, United States)	2000-2003	CC	CRC patients	641	16	482	1.7 (0.8-4.0)	Age, sex, calendar time, follow-up duration (matched)	No increased risk
			Non-CRC control	641	9	471			
Kuiper *et al*[33] 2020(Netherlands)	2007-2014	CC	CRC patients	1978	1041	937	1.08 (0.97-1.21)	Age, sex, calendar time, H2 blocker use, aspirin, NSAIDs, statins, antidiabetics use	No increased risk
			Non-CRC control	7912	4161	3751			

BMI: Body mass index; CC: Case-control; PPI: Proton pump inhibitors; CRC: Colorectal cancer; CI: Confidence interval; H2: Histamine-2 receptor; HRT: Hormone replacement therapy; NSAIDs: Nonsteroidal anti-inflammatory drugs; NR: Not reported; OR: Odds ratio.

assessed in three retrospective studies, two post-hoc analyses of randomized controlled trials (RCTs)[39-43]. These studies cumulatively examined 7065 patients and are summarized in Table 5.

Zhang *et al*[39] examined 125 patients with stage II-III rectal cancer dichotomizing them as eligible omeprazole users (EOU, 20 mg per os at least once/day for 6 d and/or 40 mg IV infusion daily during adjuvant chemotherapy) or non-EOU, and an effective omeprazole group (EOG, OME ≥ 200 mg total during the study period), or a non-EOG. The authors found that 5-year disease-free survival (DFS) was significantly decreased in the EOG *vs* non-EOG group (P = 0.032), but OS was similar among the groups (P = 0.092). Additionally, the recurrence of rectal cancer was more common in the non-EOG group than in the EOG group (31.3% *vs* 10.3%, P = 0.025). A comparison of EOU and non-EOU patients revealed similar DFS and OS at 3 and 5 years.

In a cohort of 298 patients with stage I-III CRC, Sun *et al*[40] identified 77 patients who used PPIs concurrently during adjuvant capecitabine therapy. PPI-users were found to have significantly lower 5-year recurrence-free survival (RFS) (74% *vs* 83%, P = 0.03), but similar OS (81% *vs* 78%, P = 0.7) compared to non-users. Multivariate analysis revealed similar RFS between the groups (HR = 1.65, 95%CI: 0.93-2.94, P =

Table 4 Summary of epidemiological studies assessing the effect of proton pump inhibitors exposure on the risk of developing colorectal cancer

Author, year, place	Accrual year	Study design	Grouping	Number of patients	Developed CRC	Did not develop CRC	HR (95%CI)	Adjustments	PPI use and CRC risk
Hwang *et al* 2017[34] (Korea)	2007-2013	P	PPI users	49520	Total cases (including PPI users and non-PPI users) 5304	NR	NR	Sex, age, smoking, alcohol, BMI, consumption, physical activity, type 2 diabetes, CCI score, aspirin use, metformin use, stain use, socioeconomic status	No association
			Non-PPI users	401764					
Babic *et al* 2020[35] (United States)	1988-2015	P	PPI users	13205	83	13122	0.84 (0.67-1.04)	Age, physical activity, BMI, family history of CRC, alcohol, smoking, history of lower endoscopy, caloric intake, vitamin D, calcium intake, regular aspirin use, folate intake, menopausal hormone therapy use, and red meat	No association
			Non-PPI users	162654	1172	161482			
Lei *et al* 2020[36] (Taiwan)	1999-2011	R	PPI users	45382	172	45210	2.03 (1.56-2.63)	Sex, age, year of index date, diabetes, coronary artery disease, HTN, dyslipidemia, COPD, cirrhosis, CCI, aspirin/NSAID use, statin use, antidiabetic use	Increased risk
			Non-PPI users	45382	93	45289			

BMI: Body mass index; CCI: Charlson comorbidity index; COPD: Chronic obstructive pulmonary disease; CRC: Colorectal cancer; CI: Confidence interval; HR: Hazard ratio; HTN: Hypertension; NSAID: Nonsteroidal anti-inflammatory drugs; NR: Not reported; PPI: Proton pump inhibitor; P: Prospective study; R: Retrospective study.

0.09).

Wong *et al*[41] studied PPI use with adjuvant CapeOx (capecitabine, intravenous oxaliplatin), or adjuvant FOLFOX (intravenous 5-FU, leucovorin, oxaliplatin) therapy. Of 389 patients with stage II-III CRC, 214 underwent CapeOx therapy and 175 had FOLFOX therapy. The proportions of patients taking PPI in both groups were similar (23.4% CapeOx *vs* 28% FOLFOX, P = 0.3). Comparing PPI-users and non-users, the authors found 3-year RFS to be significantly lower in CapeOx-treated PPI-users (P = 0.029) but similar between the two groups in FOLFOX-treated patients (P = 0.66). Multivariate analysis showed that PPI use was associated with increased risk of CRC recurrence in the CapeOx-treated group (HR = 2.20, 95%CI: 1.14-4.25, P = 0.018). The use of PPI in combination with either adjuvant treatment regimen did not affect 3-year OS in this study (CapeOx, P = 0.35; FOLFOX, P = 0.929).

Kichenadasse *et al*[42] analyzing data from six RCTs including metastatic CRC patients reported that PPI-users had significantly poorer OS (HR = 1.20, 95%CI: 1.03-1.40, P = 0.02) and PFS (HR = 1.20, 95%CI: 1.05-1.37, P = 0.009). The subgroup analysis revealed that chemotherapy type, use of capecitabine or 5-FU, line of therapy and VEGF inhibitor use, across studies, did not influence oncological outcomes between users and non-users. Kim *et al*[43] described post-hoc analysis of data relating to PPI use from the AXEPT trial. The authors reported that PPI users in the FOLFIRI (fluorouracil, leucovorin, irinotecan) arm had significantly better OS (HR = 0.5, 95%CI: 0.30-0.85; P = 0.011) and PFS (HR = 0.55, 95%CI: 0.33-0.91, P = 0.02) compared to non-users, while there were no differences noted in the mXELIRI (capecitabine, irinotecan) arm.

DISCUSSION

This systematic review of 26 articles is the first in the literature to summarize the evidence on the association between PPI and CRC from basic research, epidemiological, and clinical treatment studies. Previously published meta-analyses by Ahn *et*

Table 5 Summary of treatment studies

Author	Center	Study design	Cancer stage and type	Cancer treatment	PPI use (definition)	No. of patients	Results
Zhang *et al* [39] 2017	Guangzhou, China	R	Stage II-III Rectal cancer	LCRT (46 Gy, Oxaliplatin + Capecitabine (2 cycles)	EOU = OME: 20 mg PO, min. OD for 6 d / 40 mg IVI, daily). EOG = total OME dose ≥ 200 mg[1]	125	EOG *vs* non-EOG: [1]DFS (3-year) = 77.1% *vs* 96.6%, *P* = 0.032, DFS (5-year) = 69.6% *vs* 46.7%, *P* = 0.032, OS (3-year) = 82.3% *vs* 96.6%, *P* = 0.092, OS (5-year) = 76.9% *vs* 89.5%, *P* = 0.092 EOU *vs* non-EOU: [1]DFS (3-year) = 85.5% *vs* 77.8%, *P* = 0.658, DFS (5-year) = 75.6% *vs* 74.6%, *P* = 0.658, OS (3-year) = 90.3% *vs* 82.5%, *P* = 0.754, OS (5-year) = 82% *vs* 77.6%, *P* = 0.754
Sun *et al*[40] 2016	Edmonton, Canada	R	Stage I-III CRC	Adjuvant Capecitabine monotherapy	Any use during treatment (based on prescription data)	298	PPI-user *vs* non-users: RFS (5 years) = 74% *vs* 83%, *P* = 0.03; OS (5-year) = 81% *vs* 78%, *P* = 0.7. Multivariate RFS (5-year): HR (95%CI) = 1.65 (0.93-2.94), *P* = 0.09
Wong *et al*[41] 2019	Alberta, Canada	R	Stage II-III CRC	Adjuvant CapeOx or FOLFOX	Any use during treatment (based on prescription data)	389	PPI-users *vs* non-users, RFS (3-year): CapeOX = 69.5% *vs* 82.6%, *P* = 0.03; FOLFOX = 82.9% *vs* 61.7%, *P* = 0.7; Multivariate RFS: HR (95%CI) = 2.20 (1.14-4.25) *P* = 0.018; OS (3-year): CapeOX = 90.1% *vs* 91.2%, *P* = 0.345, FOLFOX = 77.4% *vs* 80.1%, *P* = 0.929
Kichenadasse *et al*[42] 2021	6 clinical trials	Retrospective post-hoc analysis of RCT	Stage IV CRC	Fluoropyrimidine-based chemotherapy (± additional agents). Regimens differed across included trials	Minimum 7 d of use during study period	5633	OS: Significantly worse in PPI-users [HR (95%CI) = 1.20 (1.03-1.40)], *P* = 0.02; PFS: Significantly worse in PPI-users [HR (95%CI) = 1.20 (1.05-1.37)], *P* = 0.009 Various treatment subgroups did not influence OS and PFS
Kim *et al*[43] 2021	China, Japan, South Korea (98 centers)	Retrospective post-hoc analysis of RCT	Stage IV CRC	mXELIRI or FOLFIRI (± Bevacizumab)	Use for ≥ 20% of study period	620	mXELIRI arm: No difference in OS or PFSFOLFIRI arm: Significantly better OS [HR (95%CI) = 0.5 (0.3-0.85), *P* = 0.11] and PFS [HR (95%CI) = 0.55 (0.33-0.91), *P* = 0.20] in PPI users

[1]Definitions of EOU and EOG are stated in the results section. CapeOX: Capecitabine plus oxaliplatin; CRC: Colorectal cancer; CI: Confidence interval; DFS: Disease free survival; EOU: Eligible omeprazole users; EOG: Effective omeprazole group; FOLFOX: 5-Fluorouracil, leucovorin, and oxaliplatin; FOLFIRI: 5-Fluorouracil, leucovorin, and irinotecan; HR: Hazard ratio; LCRT: Long-course chemoradiotherapy; mXELIRI: Capecitabine plus irinotecan; OME: Omeprazole; OS: Overall survival; PPI: Proton pump inhibitors; PFS: Progression free survival; RFS: Recurrence free survival; R: Retrospective cohort study; RCT: Randomized controlled trials.

al[12] and Ma *et al*[13] primarily focused on the epidemiological aspect, assessing the risk of CRC with PPI exposure. In this systematic review, we describe evidence from basic research studies on the potential pro-tumor (proliferative) and anti-tumor (therapeutic) effects of PPI, assess if these findings are translatable into human studies, and discuss future clinical and research aspects related to the use of PPI in patients with CRC.

Although primarily responsible for gastric acid secretion, gastrin and its precursors are also potent growth factors for normal and malignant GI tissues[44]. Gastrin exerts its trophic effect through interaction with CCKBR, resulting in activation of growth-promoting downstream pathways[24,44]. As noted, long-term PPI use causes hypergastrinemia, raising concerns regarding the effects of PPI-induced hypergastrinemia on GI cancers. Recent reviews on the association of PPI use and various GI cancers such as pancreatic, hepatocellular, esophageal, and gastric cancer have yielded conflicting evidence[45-47]. Previous reviews addressing PPI and CRC suggested that there may not be any causative association between them[12-13]. However, Ma *et al* [13] suggested that long-term PPI use (> 5 years) may increase CRC risk.

Of the six basic research studies on PPI-induced hypergastrinemia, four demonstrated that PPI did not influence CRC growth and progression, whereas two suggested that PPI may even have a protective effect against CRC[16,17,19-21]. The

two publications reporting a suggested protective effect, by Penman *et al* and Tobi *et al* [21], demonstrated a lower CRC tumor burden in PPI-treated animal models and a dose-dependent decrease in CRC cell line (NCL-H716) proliferation, respectively. These findings may be suggestive of an anti-tumor effect of this drug class or may be explained by a possible interaction with the carcinogen (azoxymethane) used for tumor induction (as Penman *et al*[20] hypothesized).

The included human epidemiological studies do not present compelling evidence of a causative relationship between PPI use and CRC. Seven of nine studies demonstrated no significant risk of CRC development in patients previously or currently using PPI[28-35]. However, of these, Lee *et al*[31] reported an increased incidence with long-term use (≥ 10 years), whereas Hwang *et al*[34] found increased cases in a specific cohort of patients using PPI and at low risk of developing CRC. Of the remaining two studies, Lei and colleagues[36], found that the risk of CRC was significantly increased in PPI users while Kuiper *et al*[33] found significantly increased risk only in current PPI users, especially those using NSAIDs concomitantly. These results from human studies may be corroborative of the basic research findings that PPI do not have a growth-promoting effect on CRC. However, two included retrospective analyses examining survival among CRC patients, found that mortality risk was significantly higher in those using PPI compared to non-users[37,38]. In their cohort, Graham *et al*[37] found any comorbidities, advanced tumor stage, and poor tumor differentiation to be significant predictors of mortality. Such data may depict a potential pro-tumor influence of PPI on the CRC microenvironment, in contrast to findings from basic research mentioned above. Another explanation for poorer survival seen in PPI-using patients with CRC could be drug-drug interactions between PPI and commonly used chemotherapeutics, such as capecitabine. However, neither study describes information on CRC treatment of their cohorts.

Capecitabine is rapidly and predominantly absorbed from the upper GI tract[48]. It is thought that the dissolution and absorption of capecitabine may be reduced with increasing gastric pH (an effect produced by PPI)[49]. Sun *et al* and Wong *et al* studied the drug interaction between PPI and capecitabine in patients diagnosed with CRC. After adjustment for confounders, these authors found conflicting evidence: Sun *et al* [40] reported similar RFS, but Wong *et al*[41] found significantly lower 3-year RFS in the cohort concomitantly treated with CapeOx and PPI. These studies did not account for several potential confounders, such as concomitant drug use (statins, aspirin, anti-diabetics), serious comorbidities, and treatment modifications, making it difficult to draw firm conclusions. Furthermore, a recent study by Sekido *et al*[50] concluded that rabeprazole does not influence the plasma concentration of capecitabine and its metabolites, and subsequently their inhibitory effect on CRC cell proliferation.

Several basic research studies have also focused on identifying potential anti-tumor mechanisms of PPIs in CRC. Three basic research studies revealed that PPI may exert anti-tumorigenic effects through several mechanisms: Reducing pro-inflammatory signaling molecules (TNF-α, COX-2, and IL-6), oxidative stressors (NO and TBA-RS), and proteolytic enzymes (MMP-9, MMP-11, and MT1-MMP); exerting anti-mitogenic effects (inhibition of MAPKs) and anti-angiogenic effects (hypoxia-inducible factor 1-alpha, vascular endothelial growth factor, platelet-derived growth factor, IL-8); and inducing apoptosis *via* upregulating pro-apoptotic molecules (p21waf1/cip1) and downregulating anti-apoptotic molecules (cyclin A, Bcl-2, Bcl-xl and survivin)[23,24]. Additionally, these studies also found that PPI could exert anti-tumor properties even when co-administered with gastrin. It seems that instead of enhancing the trophic effects of gastrin, PPI may paradoxically inhibit these effects by interfering with the interaction between gastrin and CCKBR[24].

These results may explain the earlier findings of Penman *et al*[20] and Tobi *et al*[21], who also used omeprazole in their work and found a protective effect of PPI on CRC. Zeng *et al*[26] and Zheng *et al*[27] identified another potential action of specific PPI agents (pantoprazole, ilaprazole), as inhibitors of TOPK, a kinase highly expressed in rapidly proliferating tissues of embryological and cancerous origin[51]. The overexpression of TOPK in cancers has been associated with aggressive tumor behavior and poor clinical outcomes. Therefore, this kinase has been speculated to be a viable target for inhibiting downstream growth-promoting pathways[51]. Considering that no specific TOPK-inhibiting drugs have been approved for clinical use and the reported findings, further examination of these properties of pantoprazole and ilaprazole may be worthwhile.

It has also been suggested that PPIs have chemosensitizing ability. This was highlighted by Wang *et al*[25] demonstrating that pantoprazole enhanced the cytostatic effect of FOLFOX in CRC. Chemoresistance has been associated with an acidic tumor microenvironment, which results from the increased production of lactic acid

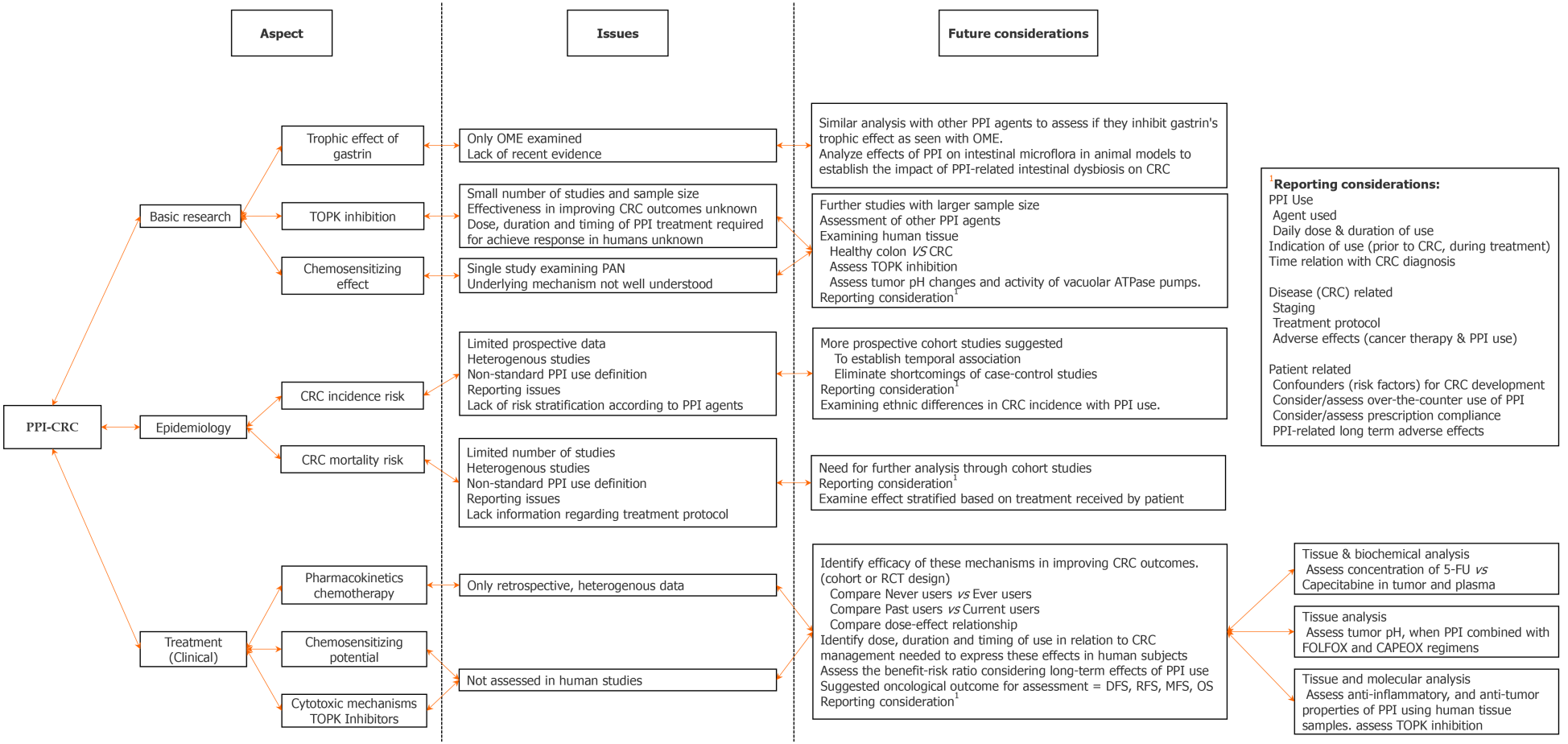

Figure 1 Figure outlining areas for future research to establish a better understanding of the relationship between proton pump inhibitors and colorectal cancer. CapeOx: Capecitabine plus oxaliplatin; CRC: Colorectal cancer; DFS: Disease free survival; FOLFOX: Fluorouracil, leucovorin, oxaliplatin; 5-FU: 5-flourouracil; MFS: Metastasis free survival; OME: Omeprazole; OS: Overall survival; PAN: Pantoprazole; PPI: Proton pump inhibitors; RCT: Randomized controlled trials; RFS: Recurrence free survival; TOPK: T lymphokine–activated killer cell-originated protein kinase.

(Warburg effect) and/or overexpression of vacuolar-ATPase pumps[52,53]. It is thought that this microenvironment neutralizes the effects of chemotherapeutic agents while decreasing their uptake into cancer cells. PPI appear to inhibit the activity of vacuolar-ATPase pumps, thereby increasing the pH of cancer cells and sensitizing them to chemotherapeutics[54]. This mechanism may suggest a potential role for PPI as adjuvants during chemotherapy, not only for the symptomatic treatment of side

effects but also to improve oncological outcomes. Zhang *et al*[39] further provided evidence to support this rationale by demonstrating lower recurrence rates and better chemoradiotherapy efficacy in patients using omeprazole concomitantly during chemotherapy compared with those did not.

Various PPI agents have been developed on the basis of the prototype PPI, omeprazole. They all share structural similarities and are generally effective and safe in the treatment of acid-related disorders[6]. However, differences in pharmacokinetics and pharmacodynamics exist among them, with the newer agents offering several advantages[55]. Although these differences are primarily related to the onset of action and degree of acid suppression, they also include reduced potential for drug interaction and other potential mechanisms of action that could make them effective in the treatment of diseases other than acid-related disorders.

Finally, it is important to mention that long-term PPI use also results in intestinal dysbiosis, with reduced abundance and diversity of gut microbiota and an increase in pathogenic bacteria[56]. Pathogenic bacteria implicated in the carcinogenesis of CRC, such as *Fusobacterium nucleatum*, *Escherichia coli*, *Enterococcus faecalis etc.* are more prevalent in PPI users[57]. These bacteria form a special microenvironment in the colorectal tissue that is conducive to neoplastic transformation and progression. They produce toxins that can damage the intestinal cell barrier, dysregulate immune cell function, induce a chronic inflammatory state, and cause DNA damage and genomic instability, all of which increase cell proliferation and contribute to the development of CRC[57,58]. One group has found that the abundance of *Fusobacterium nucleatum* may be associated with chemoresistance in CRC, resulting in poor response to 5-FU and oxaliplatin and higher recurrence rates[59]. This review did not identify any studies assessing the effect on CRC of intestinal dysbiosis resulting from PPI use. Studies focusing on the interaction of PPI-induced dysbiosis in CRC are needed to resolve the inconsistencies between the basic research and human studies.

A major limitation of this review is the heterogeneity among the included studies. The basic research studies describe experiments with different animal models and cell lines, using different PPI doses and exposure periods, whereas the human (epidemiological and treatment) studies varied in accounting for potential confounding factors and inclusion criteria for PPI exposure. Additionally, most of the human studies used prescription databases to ascertain PPI use, which may fail to accurately determine PPI use because they do not account for prescription non-adherence and possible over-the-counter use. Moreover, stratification of epidemiological and treatment-related evidence based on the individual PPI agents was lacking in all but one included study. These limitations make it difficult to present conclusive evidence on the question of whether PPI are an adversary or an ally in relation to CRC. Nonetheless, this review is the first to systematize the entirety of current evidence on the topic, and summarize data from different levels and aspects of the relationship.

In light of the evidence, we suggest that PPI use should continue when appropriately indicated, while a cautious approach should be implemented when combining them with capecitabine-based chemotherapy. Patients must be educated regarding the potential adverse effects of long-term PPI use and advised to avoid over-the-counter use for improper indications, with physicians being more diligent not to overprescribe. There are several aspects of this relationship which require further, high-quality investigation as outlined in Figure 1.

CONCLUSION

In conclusion, this review highlights an unexpected potential beneficial role of specific PPI agents in relation to CRC. First, PPI instead of promoting CRC growth *via* trophic effects of hypergastrinemia, may paradoxically inhibit them. Second, current evidence suggests that individual PPI agents may affect CRC differently: Pantoprazole and ilaprazole as TOPK inhibitors; rabeprazole with lower drug interaction capability with capecitabine; and pantoprazole and rabeprazole with little impact on CRC incidence (as evidenced by Lei *et al*[36])[26,27,50]. These findings warrant further studies to better understand these mechanisms and possibly facilitate use of PPI differently in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Proton pump inhibitors (PPI) are one of the most widely used medications globally. Several reports have raised concerns that they may be inappropriately or even overused. Several adverse effects of PPI have been reported such as increased risk of gastrointestinal cancers including colorectal cancer (CRC).

Research motivation

There is no systematic review covering the entire body of evidence on the influence of PPI on CRC carcinogenesis. Previous reviews have primarily focused on the epidemiological aspect, in terms of CRC incidence, of their relationship. A comprehensive review analyzing the association between PPI use and CRC may yield findings, which may have practical implications. Therefore, this systematic review aimed to summarize evidence from basic research studies on potential mechanisms of PPI, as well as from human epidemiological and clinical studies assessing the influence of PPI use on survival and treatment outcomes of CRC patients.

Research objectives

To summarize evidence from basic research, epidemiological and clinical studies focusing on the relationship between PPI and CRC.

Research methods

This systematic review performed according to the PRISMA guidelines was based on patients, interventions, comparisons, and outcomes. Using a predetermined search strategy, MEDLINE, EMBASE, Scopus, and Web of Science electronic databases were searched from inception until May 17, 2021. The initial search returned 2591 articles. Twenty-eight studies were included in this review and categorized as basic research studies (n = 12), epidemiological studies (n = 11), and CRC treatment studies (n = 5). The Newcastle-Ottawa Scale or Cochrane Risk of Bias 2.0 tool were utilized to assess the quality of the included studies depending on the study design.

Research results

Basic research studies show that PPI may paradoxically inhibit the trophic effect of gastrin rather than stimulating CRC development through it. Additionally, PPI may possess several anti-tumor properties (omeprazole, pantoprazole) while also being potential T lymphokine-activated killer cell-originated protein kinase inhibitors (pantoprazole, ilaprazole) and chemosensitizing agents (pantoprazole). Based on data from epidemiological studies, it appears that no causal association between PPI use and increased CRC risk exists. Treatment studies suggest that concomitant use of PPI with capecitabine use may reduce the efficacy of chemotherapy and result in poorer oncological outcomes. These studies also suggest that pantoprazole may have a chemosensitizing effect with the FOLFOX regimen.

Research conclusions

This systematic review identifies an unexpected inhibitory effect on CRC carcinogenesis by way of several potential mechanisms. Moreover, it appears that different PPI agents may have differential effects on CRC treatment, which may have practical implications. Further prospective studies are warranted to delineate this relationship as well as assess the role of individual PPI agents.

Research perspectives

PPI do not appear to have a growth promoting effect on CRC, however, a cautious approach should be adopted while concomitantly administering PPI and capecitabine-based chemotherapy. Recent evidence suggests that individual PPI agents have a differential effect on CRC carcinogenesis, with newer agents such as pantoprazole, ilaprazole and rabeprazole possessing beneficial characteristics, which may have a role in the treatment of CRC.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 28; 27(44): 7734-7738

DOI: 10.3748/wjg.v27.i44.7734

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

LETTER TO THE EDITOR

SARS-CoV-2 infection in liver transplant recipients: A complex relationship

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Author contributions: All authors contributed to the conception, writing, and review of the article and approved the submitted version.

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

Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), No. 19/02679-7 and No. 20/13148-0.

Country/Territory of origin: Brazil

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

The recent manuscript reviewed investigations involving liver damage in coronavirus disease 2019 (COVID-19) patients, and COVID-19 in patients with previous chronic hepatological diseases, such as patients with liver graft. The literature presents several conflicting results concerning the anti-SARS-CoV-2 response in patients with solid organ transplants, in liver transplant recipients. Therefore, we would like to humbly state a few points for consideration involving liver transplant recipients and COVID-19, such as the time since transplantation, comorbidities, and immunosuppressive regimens.

Key Words: COVID-19; SARS-CoV-2; Liver transplant; Immunosuppression; Infection; Comorbidities

Core Tip: There is not a consensus whether solid organ transplant recipients present increased severity or death rates due to coronavirus disease 2019 (COVID-19) compared with the general population. In particular, liver allograft has a low risk of rejection, therefore enabling treatment with relatively less immunosuppressive regimens. The reduction in the production of proinflammatory cytokines, without a drastic suppression of the immune response, may benefit liver transplant recipients during COVID-19. Further investigations should compare different organ transplant

Received: July 20, 2021
Peer-review started: July 20, 2021
First decision: August 6, 2021
Revised: August 14, 2021
Accepted: November 17, 2021
Article in press: November 17, 2021
Published online: November 28, 2021

P-Reviewer: Han J
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

recipients, elapsed time from the organ transplant, different immunosuppressive treatments, and their anti-SARS-CoV-2 response.

Citation: Alberca RW, Benard G, Alberca GGF, Sato MN. SARS-CoV-2 infection in liver transplant recipients: A complex relationship. *World J Gastroenterol* 2021; 27(44): 7734-7738
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7734.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7734

TO THE EDITOR

We read with great interest the article entitled "Liver dysfunction and SARS-CoV-2 infection", recently published by Gracia-Ramos *et al*[1] in the World Journal Gastroenterology[1]. Gracia-Ramos *et al*[1] performed a review on patients with liver dysfunction and coronavirus disease 2019 (COVID-19), highlighting investigations involving liver injury in COVID-19 patients, and COVID-19 in patients with previous chronic liver disease, such as cirrhosis and liver transplant recipients. Nevertheless, we would like to raise a few considerations regarding liver transplant recipients and COVID-19.

Transplantation is a treatment for organ failure and end-stage organ illnesses, requiring patients to undergo regular use of immunosuppressive treatment to avoid organ rejection[2]. There is no consensus regarding the increase in the incidence or severity of COVID-19 on solid organ transplant (SOT) recipients[3]. A few reports have identified an increase in severe COVID-19 and mortality rate in SOT recipients[4,5], while others failed to do so[3,6].

SOT patients may respond differently to COVID-19 due to associated comorbidities, the organ grafted, elapsed time from the transplant surgery, drugs used to prevent organ rejection, or drugs used to treat SARS-CoV-2 infection[7-9]. The identification of the organ grafted is usually described in the manuscripts[6], but only one manuscript with comparison between the different organ transplant recipients has been made so far, identifying an increase in mortality in kidney and heart transplant recipients in comparison to liver transplant recipients[5].

A systematic review identified a similar death rate in liver transplant recipients and non-transplant recipients, but a higher percentage of non-transplant recipients were obese or had cardiovascular or respiratory diseases[10]. Hospitalization in the intensive care unit presented mixed results, and only one investigation reported the need for mechanical ventilation, and liver transplant recipients presented a greater need for it, in comparison to non-SOT patients[10]. Consequently, it is not possible to confirm if liver transplant recipients have a lower risk for severe illness or death risk in comparison to non-transplant recipients during COVID-19.

The liver allograft has a lower risk of rejection in comparison to heart and kidney allograft. Therefore, it is usually treated with reduced immunosuppressive regimens compared with the other SOT patients[11,12]. Different immunosuppressive regimens can affect the detection of SARS-CoV-2 RNA in nasopharyngeal swab tests[13]. Tacrolimus, a commonly used drug by SOT patients, has been shown to display anti-coronavirus effects in vitro[14] and putative protective properties in liver transplant recipients with COVID-19[15]. In contrast, another report identified that tacrolimus has no significant effect on the mortality risk[16].

We hypothesize that the inhibition of the calcineurin by tacrolimus could curb the production of proinflammatory cytokines, similarly to the observed in other inflammatory models[17-19], and reduce or prevent the development of the cytokine storm in COVID-19[20]. A recent report associated tacrolimus therapy with a protective effect in liver transplant recipients with COVID-19[15]. Importantly, the use of tacrolimus in association with dexamethasone in immunocompetent COVID-19 patients is currently being tested in a Spanish clinical trial (clinicaltrials.gov/ct2/show/NCT04341038).

Importantly, SOT recipients (including liver, kidney, heart, lung, and others) have an increased risk of mortality due to infections in the first year after the transplant[21], especially respiratory infections[22]. A recent review identified a higher mortality rate in patients with over a year of organ draft[23]. Another report identified no difference in elapsed time from transplant in survival and non-survival COVID-19 patients, but

patients presented a high incidence of other comorbidities[24]. In a recent report, liver transplant was not associated with greater mortality risk, while the association with other comorbidities (mainly diabetes, hypertension, obesity, and cardiovascular disease) posed a higher death risk[25]. It is reasonable to assume that comorbidities associated with poor outcomes in non-SOT patients will also influence the COVID-19 disease course in liver recipients, such as co-infection[26], respiratory disorders[27], and alcohol consumption[28]. In another report, liver transplant recipients presented similar mortality to non-SOT patients and reduced mortality in comparison with patients with liver cirrhosis[29].

On the other hand, liver transplant recipients developed lower levels of anti-SARS-CoV-2 IgG, and a more pronounced reduction in SARS-CoV-2-specific IgG levels, 6 mo after COVID-19[30]. Therefore, a long-term follow-up is necessary to fully determine the duration of the anti-SARS-CoV-2 immune response and the long-term protection offered by COVID-19 vaccines in liver transplant recipients. A recent report has identified SARS-CoV-2 infections in fully vaccinated SOT patients, and, importantly, the death of a fully vaccinated heart transplant patient due to COVID-19[30].

In conclusion, Gracia-Ramos *et al*[1] presented interesting points concerning liver transplant recipients with COVID-19. However, further investigations are needed to better understand the impact of comorbidities, elapsed time since the organ transplant, immunosuppressive regimen, and vaccination on COVID-19 in liver transplant patients.

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