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Secondary causes of inflammatory bowel diseases

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Abstract

Inflammatory bowel diseases (IBD), conventionally consist of Crohn's disease (CD) and ulcerative colitis. They occur in individuals with high risk genotype for the disease in the setting of appropriate environmental factors. The pathogenesis of IBD involves a dysregulated autoimmune response to gut dysbiosis, which in turn is triggered due to exposure to various inciting environmental factors. But there is no clearly defined etiology of IBD and this type of disease is termed as "idiopathic IBD", "classic IBD", or "primary IBD". We reviewed the current medical literature and found that certain etiological factors may be responsible for the development of IBD or IBD-like conditions, and we consider this form of *de novo* IBD as "secondary IBD". Currently known factors that are potentially responsible for giving rise to secondary IBD are medications; bowel altering surgeries and transplantation of organs, stem cells or fecal microbiome. Medications associated with the development of secondary IBD include; immunomodulators, anti-tumor necrosis factor alpha agents, anti-interleukin agents, interferons, immune stimulating agents and checkpoint inhibitors. Colectomy can in some cases give rise to *de novo* CD, pouchitis of the ileal pouch, or postcolectomy enteritis syndrome. After solid organ transplantation or hematopoietic stem cell transplantation, the recipient may develop *de novo* IBD or IBD flare. Fecal microbiota transplantation has been widely used to treat patients suffering from recurrent *Clostridium difficile* infection but can also causes IBD flares.

Key words: *De novo* inflammatory bowel disease; Secondary inflammatory bowel disease; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Core tip: Inflammatory bowel diseases (IBD) are chronic illnesses of the gastrointestinal tract with no clearly defined etiology and are traditionally termed as primary IBD. It is generally believed that IBD results from abnormal immune response to dysbiosis of gut microbiota in a genetically susceptible individual. IBD or IBD-like conditions may also be caused by well-defined etiologies; such as medical, surgical, and organ transplantation. These conditions are coined as secondary IBD. In this review we attempted to highlight some etiological factors, pathogenetic pathways, and clinical features of secondary IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are idiopathic chronic diseases of the gastrointestinal (GI) tract that are traditionally divided into ulcerative colitis (UC) and Crohn's disease (CD) based on their respective phenotypic presentation. Sometimes there is an overlap in clinical presentation, colonoscopic findings and histopathological features between UC and CD; which is termed as indeterminate colitis^[1,2]. UC is the most predominant type of IBD with a prevalence of 7.6 to 246.0 cases per 100000 per year, followed by CD which has a prevalence of 3.6 to 214.0 cases per 100000 per year^[3]. The worldwide distribution of IBD is skewed towards being more prominent in North America and Europe, although in the past two decades its prevalence has risen in developing countries like China and India^[4,5]. This change in trends has paralleled with changes in dietary habits like inclusion of processed foods, increased intake of sugars and fats, overutilization of antibiotics and an overall improvement in hygiene.

The diagnosis of IBD is made by correlating clinical presentation, endoscopic findings and histopathological features of diseased tissue specimens. There is no single test to diagnose IBD or to distinguish between the CD and UC, although use of perinuclear anti-neutrophil cytoplasmic antibody and anti-saccharomyces cerevisiae antibody titers can sometimes be helpful in distinguishing the two^[6]. Gut inflammation in UC is limited to the mucosal layer (epithelium, lamina propria and muscularis mucosa) and may extend up to the superficial submucosa. On the other hand, CD is characterized by the presence of non-caseating granulomas, transmural inflammation of the gut and formation of strictures and fistulas. In rare circumstances, CD can manifest solely as a perianal disease without bowel involvement^[7]. The main differentiating features distinguishing CD from UC are the presence of granulomas, transmural disease, rectal sparing, and formation of strictures and/or fistulas. Although UC can occasionally manifest with strictures and perianal abscess or fistulas^[8], Classic UC is expressed as a contiguous inflammation almost always involving the rectum and extending proximally to the left colon or entire colon, *i.e.*, extensive colitis or pancolitis. Sometimes the disease can extend into distal 10-15 cm segment of the terminal ileum, which is termed as backwash ileitis. CD on the other hand can arise in any part of the GI tract with a segmental distribution. It usually involves the ileocecal region, sparing the rectum. Smoking is considered as a risk factor for CD, but a protective factor for UC. These features of UC and CD suggest that their etiopathogenesis may not completely overlap. In addition, there are extra-intestinal manifestations such as erythema nodosum, pyoderma gangrenosum, and primary sclerosing cholangitis (PSC).

The etiology and pathogenesis of IBD remain unclear with several speculations suggesting the role of genetic factors, gut microbiome, and immune dysregulation. The interaction between these aforementioned factors gives rise to various immunogenic types and clinical phenotypes of disease state. We term this type of traditional disease state as "idiopathic inflammatory bowel disease", "classic inflammatory bowel disease" or "primary inflammatory bowel disease" due to its unclear or idiopathic etiology and pathogenesis. However, certain identifiable factors like medications, bowel-alternating surgery, and transplantation of organs, stem cells or fecal microbiota appear to induce IBD or IBD-like changes in the GI tract. The affected individuals

essentially present with the fitting clinical symptoms and signs supported by endoscopic, histopathologic, and radiologic findings of IBD. We have termed this specific type of disease as “secondary inflammatory bowel disease (SIBD)” (Table 1).

Pathogenesis of conventional IBD

Genetic mutations or acquiring variants of certain genes have been proposed to be a pre-disposing risk factor for developing IBD. Genetic alteration in the gene coding for nucleotide-binding oligomerization domain 2 has been found to be associated with CD in about 20% of cases^[9]. This mutation is associated with reduced response to bacterial lipopolysaccharides leading to increased survivability of certain gram negative bacteria that translocate into the bowel epithelium and induce inflammation. A loss of function mutation in the alleles coding for fucosyltransferase 2 and thereby absence of secretion of this enzyme in the intestinal tract is associated with an increased risk of alterations in the microbiome^[10]. Genetic defects leading to abnormal T-cell function and macrophage activity can induce immune-mediated gut injury^[11]. These cellular alterations induce dysregulated cytokine production and release, which recruits more inflammatory cells and continues the process of immune-mediated inflammatory response. Genetic linkage analysis has shown that mutations in the IL-10/IL-10R signaling pathway has been associated with infancy or early childhood onset IBD^[12,13].

The GI tract along with the mesentery consist of a vast number of immune cells making it a highly immunogenic organ. The gut microbiome lives in harmony with the host defense system that protects the host from invasive GI pathogens. This tolerance to the gut microbiome is mediated by the homeostasis of intestinal microbiota, gut epithelial cell, stromal cells of the intestines, antigen presenting cells (dendritic cells, tissue macrophages) and inflammatory cells (neutrophils, lymphocytes)^[14]. Alteration in the gut microbiome and a dysregulated response by any of these cells can shift the delicate balance of host defense and immune tolerance leading to development of IBD^[15]. There is decreased biodiversity in the microbiome of individuals diagnosed with IBD^[16]. Although a study in monozygotic twins showed the opposite results among individuals diagnosed with CD^[17].

Dysbiosis plays an important role in the pathogenesis of classic IBD. The *Phyla Firmicutes* and specifically the family of Gram negative enteric organisms, *i.e.*, *Enterobacteriaceae*, have been found to be abundant in the diseased state of IBD^[18,19]. Short-term antibiotic therapy has shown to improve gut inflammation, likely by affecting gut bacteria and regulating dysbiosis^[20]. Another mechanism of microbial effect on the GI tract is the ability of the gut bacteria to adhere to mucosal surface and invade the deeper submucosal layers inducing an inflammatory reaction^[21,22]. Subsequently mucosal breakdown occurs due to inflammatory cell-mediated tissue injury and the damaged mucosa further exposes the sub-epithelium to more colonies of bacteria leading to a vicious cycle of antigenic exposure and mucosal injury.

In short, the pathogenesis of IBD involves a dysregulated autoimmune response to gut dysbiosis which is precipitated by exposure to environmental factors among individuals who have a pre-existing high-risk genotype. Proinflammatory factors like tumor necrosis factor (TNF) alpha, interleukins-12/23 and cell adhesion molecules (integrins/intercellular adhesion molecules) play a key role in immune activation and recruiting immune cells^[23]. Modern biological therapy has been designed to block these mechanisms or pathways, with TNFα blockers like infliximab and adalimumab being examples. These agents have also been effective in treating other autoimmune or rheumatological conditions. Interestingly, IBD often coexist with some of the systemic autoimmune disorders, and some of them are classified as extra-intestinal manifestations of IBD. In addition, genetic linkage analyses have shown an overlap of mutations in gene loci for IBD and these other autoimmune conditions^[24]. These conditions include ankylosing spondylitis, lupus and rheumatoid arthritis. There could be a common pathogenic mechanism or a relationship at genomic level existing between IBD and other immune-mediated conditions.

DRUG-INDUCED SECONDARY IBD

Environmental factors influence the disease course in IBD patients. Two such well-studied factors are smoking and appendectomy, which are associated with an increased risk of developing CD, and a decreased risk for UC^[25]. However, there are several other factors highlighted in this article that appear to induce IBD (Table 1). The pathogenesis of IBD is centered on dysregulated immunity, as described earlier. Hence it is not surprising to find that medications which alter the host immunity can

Table 1 Classification of secondary inflammatory bowel diseases based on etiology

Classification	
Drug-induced secondary IBD	Immunomodulators: Azathioprine, 6-mercaptopurine, tacrolimus, mycophenolic acid, cyclosporine; Anti-TNF agents: Infliximab, adalimumab, etanercept; Anti-interleukin agents: Secukinumab, tocilizumab; Interferons: Interferon α ; Immune stimulating agents: GM-CSF (sargramostim), G-CSF (filgrastim); Checkpoint inhibitors: Ipilimumab, nivolumab, pembrolizumab
Post-surgical secondary IBD	Post-colectomy enteritis syndrome; Post-colectomy ileal pouchitis; Post-colectomy <i>de novo</i> Crohn's Disease of the Pouch; Post-bariatric surgery: Roux-en-Y gastric bypass
Post-transplant secondary IBD	Fecal microbiota transplantation related IBD; Post- hematopoietic stem cell transplant IBD: cord colitis; Post-solid organ transplant IBD: liver, kidney

IBD: Inflammatory bowel disease; GM-CSF: Granulocyte monocyte-colony stimulating factor; G-CSF: Granulocyte-colony stimulating factor.

sometimes lead to the development of *de novo* IBD, which we have termed as “drug-induced secondary IBD”^[26]. These medications mainly include immunomodulators and biological agents. A newer class of drugs called the checkpoint inhibitors used in treating melanoma and other malignancies have also been implicated in precipitating IBD. There is a similar but weak association with the use of immune stimulating agents as well.

Immunomodulators

Immunomodulators alter the immune system mainly by inhibiting lymphocyte function. These medications consist of, but are not limited to azathioprine, 6-mercaptopurine (6-MP), tacrolimus, cyclosporine A, and mycophenolate mofetil (MMF). They are commonly used to prevent graft rejection after kidney and liver transplantation. Azathioprine and 6-MP have a well-established role in achieving long-term disease remission in the management of IBD. Cyclosporine A has also been used for the treatment of refractory IBD, including during acute UC flares^[27].

Immunomodulators may induce IBD or IBD-like conditions. Studies have shown that the post-organ transplant use of immunomodulators causes a down-regulation of regulatory T-cells in the colonic mucosa^[28]. This may create a propensity to develop immune-mediated inflammation in the colon, as regulatory T-cells prevent activation of B and cytotoxic T-lymphocytes. Tacrolimus is a routinely used immunosuppressant for organ transplantation due to its predictable side-effect profile and the availability of tests for monitoring its serum level. The use of tacrolimus has been reported to induce a flare of pre-existing IBD among individuals with solid organ transplantation^[29]. Its use has also been shown to be associated with the development of *de novo* IBD^[29,30]. In an observational study of 53 patients without a diagnosis of IBD who underwent liver transplantation, 6 (11%) of them developed *de novo* IBD during a median follow up of 3.9 years^[29].

The use of MMF as an immunosuppressant is mainly seen in kidney or liver transplant recipients. One of its common side effects is diarrhea, and in about 9% of cases it causes “MMF-induced colitis”^[31,32]. This type of colitis presents with IBD-like features of endoscopic and histologic changes in the colon^[31]. The colonoscopic appearance of MMF-induced colitis is similar to that seen in classic IBD or graft-versus-host disease. But it exhibits mucosal eosinophilic predominance with the lack of apoptotic microabscesses and endocrine cell aggregates in lamina propria on histology^[33]. This disease can sometimes persist even after discontinuation of MMF, taking up to 4-6 m for endoscopic resolution^[34]. There have been multiple reported cases of individuals who developed *de novo* CD after exposure to MMF, followed by improvement of disease after stopping the medication^[32,35,36]. There was a reported case of rapid resolution of MMF-induced colitis with a single dose of infliximab, suggesting the role of TNF in its pathogenesis^[37]. One can speculate that MMF-induced colitis represents a variant of IBD. Paradoxically, there is some evidence to support the use of MMF for treating active IBD^[38]. In a study of 25 patients with steroid-dependent disease unresponsive to biologics, MMF therapy achieved a clinical response in nearly 50% of cases^[39]. Two small studies have shown that MMF induced steroid-free clinical remission in about 25% of cases^[39,40]. Its therapeutic effect may help achieve long-term disease remission^[41]. Therefore it appears to be effective in treating IBD while it can induce colitis resembling IBD or lead to SIBD^[42]. This shows that interference with our complex immune system may be beneficial or detrimental for either treatment or induction of disease.

Anti-TNF α agents

Anti-TNF α inhibitors are molecules directed against the proinflammatory TNF α and they alter TNF α -mediated immune signaling in inflammatory pathways. Some examples of these agents are infliximab, adalimumab, and etanercept. They have been used for treating various immune-mediated rheumatological disorders, IBD, or IBD-related extra-intestinal manifestations, such as ankylosing spondylitis, uveitis, erythema nodosum, and pyoderma gangrenosum^[43]. Treating one autoimmune condition can sometimes precipitate another due to alteration in the immunological homeostasis. Interestingly, individuals who are exposed to an anti-TNF agent, may develop *de novo* IBD or a form of drug-induced SIBD^[44,45]. Along the same lines, new-onset psoriasis or eczema has been reported after exposure to the anti-TNF agents^[44,46]. For example, the administration of infliximab for the treatment of IBD-related and non-IBD related inflammatory arthropathies has shown to induce autoimmune conditions like drug-induced lupus, autoimmune hepatitis and multiple sclerosis^[47-49]. In some cases a paradoxical response is encountered after its administration, where previously arthritis-free individuals with IBD develop new-onset IBD-related arthropathy^[50].

Etanercept, an anti-TNF α agent, has been extensively used to treat rheumatoid arthritis, while being ineffective in treating IBD. In fact, the use of this agent has shown to be associated with development of *de novo* IBD in multiple clinical observations^[45,51-53]. In a large case series of patients who received etanercept for various rheumatological disorders, 49 individuals developed *de novo* IBD^[52]. The average duration of therapy before the onset of symptoms was 3.58 mo. Its use has also been associated with precipitating pre-existing UC^[51]. In a French study, the average frequency of etanercept-related *de novo* IBD was approximately 0.15%. In the same study, two patients who were treated with infliximab developed IBD^[45]. In another large study of 17018 patients with auto immune disorders, several patients were on etanercept, infliximab, or adalimumab. The ones on etanercept showed a significantly increased risk of developing *de novo* UC or CD, yet no such effect was seen with infliximab or adalimumab^[53]. In a case report, a 56-year-old male with psoriasis who was treated with adalimumab developed *de novo* UC^[54]. His UC responded to the anti-IL-12/ 23 agent, ustekinumab.

The phenomenon of paradoxical response to agents like infliximab, adalimumab, or etanercept leading to the development of *de novo* IBD needs to be further explored. We hope that ongoing research may be able to identify individualized pathogenetic pathways for each patients which will aid in selecting their appropriate therapeutic agent, and pave the way to personalized medicine in IBD care. Another emerging aspect of IBD therapy is the use of biosimilars, which needs to be further studied in regard to their ability to induce *de novo* IBD resulting in SIBD.

Anti-interleukin agents

Anti-TNF α agents like infliximab and adalimumab have become the preferred therapies for treating IBD. Recently an anti-interleukin agent, ustekinumab (anti-IL-12/23) has been approved for the treatment of CD and UC^[55,56]. The agent has been successfully used for treating psoriasis and other rheumatological conditions for the past decade. Other anti-interleukin agents have been created for their anti-inflammatory effects; and these include, but are not limited to; secukinumab, an anti-IL-17A agent used for treating psoriasis, and tocilizumab an anti-IL-6R inhibitor used for treating rheumatoid arthritis^[57,58]. In a separate study, both these agents have been found to be associated with exacerbation of pre-existing IBD^[59,60]. These agents may even induce *de novo* IBD in individuals at risk. Therefore we recommend that new agents, which could have the potential for therapy in immune-mediated disorders like IBD, need to be thoroughly studied for the immunogenicity and their association with the development of *de novo* IBD.

Interferon

Interferons (IFNs) are glycoprotein molecules which are secreted by host cells in response to viral infections and act as cytokines in inflammatory cascades. They have a role in inhibiting viral replication by inducing an immunological response through the activation of antigen-presenting cells, natural killer cells, neutrophils, and lymphocytes. IFNs have been investigated as potential immunological agents for treating IBD, but the studies showed no considerable clinical benefits^[61-63]. IFN α has been used for treating hepatitis B or hepatitis C infection. With the current use of highly effective direct acting antiviral agents, the use of IFNs has been largely out of practice. Nevertheless, IFNs are still used for treating multiple sclerosis, certain types

of lymphomas, and leukemias.

There have been multiple reported cases of chronic hepatitis C-infected individuals who developed *de novo* UC after receiving IFN α -based therapy^[64-66]. There was one reported case in which CD developed after IFN therapy for an HCV and HIV co-infected individual^[67]. All of these reported cases responded to 5-aminosalicylic acid compounds or corticosteroids. There have been several reported cases of UC flare during IFN therapy for chronic HCV infection^[68,69]. IFN α has a tendency to stimulate T helper-1 cells which play a key role in the pathogenesis of several immune-mediated disorders^[70]. This immune response in the gut may be responsible for the development of *de novo* IBD.

Immune stimulating agents

It has been proposed that CD in some individuals is related to a state of immune deficiency rather than over-activity. The immunodeficiency hypothesis can be used to explain the higher propensity of developing IBD among individuals with inherited immune disorders like Wiskott-Aldrich syndrome and glycogen storage disorders^[71]. Cellular cytokines like granulocyte monocyte-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor stimulate the hematopoietic stem cells in the bone marrow to induce production and maturation of granulocytes and monocytes, which play a role in innate immunity. Animal studies have shown that mice with an increased level of anti-GM-CSF antibodies had a reduced neutrophilic phagocytic capacity. The defect in the innate mucosal defense mechanism of the gut can lead to an increased risk of developing bowel inflammation^[72]. The deficiency in GM-CSF along with nucleotide-binding oligomerization domain 2 mutations, impairs the innate immune response resulting in bacterial invasion of the lamina propria, and these bacteria in turn stimulate gut-specific T-lymphocytes^[9]. These activated T-cells produce several inflammatory markers which may lead to development of IBD among predisposed individuals.

Several lines of clinical evidence suggest an association between GM-CSF and IBD. An Australian study of patients with IBD found that the level of antibody to GM-CSF was higher in the CD cohort than that in the UC cohort^[73]. The higher levels were associated with more penetrating or stricturing disease, which in turn is associated with an increased risk of bowel surgery. Overall, the antibody level was significantly higher in the IBD group than the control group. The presence of anti-GM-CSF antibody suggests a disease state and the antibody titer could be used as a prognostic marker for disease activity, especially in case of CD^[74]. A familial association with this antibody production has been described among those who have a family history of UC or CD^[75]. The anti-GM-CSF antibody was found to be associated with an increased intestinal permeability and bacterial translocation, which was in turn correlated with a higher likelihood of developing IBD flares^[76]. The use of immune stimulating therapy has been proposed for the treatment IBD. The use of recombinant GM-CSF (sargramostim) and granulocyte-colony stimulating factor (filgrastim) for treating CD has shown some therapeutic effect^[77,78]. The studies demonstrated an improved steroid-free survival and reduction in the Crohn's disease Activity Index score^[78,79].

Among individuals with CD, low immunoglobulin levels have been associated with an increased risk for undergoing surgeries^[80]. Based on immunodeficiency hypothesis, individuals with lower immunoglobulin levels are at risk for developing severe CD. We can also speculate that such individuals could be susceptible for developing *de novo* IBD or IBD-like conditions in the presence of underlying high-risk genotype. Intravenous immunoglobulin therapy is used for various autoimmune disorders like myasthenia gravis, systemic sclerosis, and multiple sclerosis^[81-83]. Studies have also demonstrated therapeutic benefits of IVIG in treating refractory IBD^[84,85].

Checkpoint inhibitors

Checkpoint inhibitors include, but are not limited to, ipilimumab, nivolumab, and pembrolizumab. These medications are used for treating multiple malignant conditions including melanoma^[86]. Ipilimumab is a monoclonal antibody directed against the cytotoxic T-lymphocyte antigen-4 which helps in downregulating the T-cell function^[87]. Nivolumab and pembrolizumab are monoclonal antibodies which act on the programmed cell death-1 receptor. Studies have shown that approximately 30% of individuals treated with ipilimumab develop IBD-like gut inflammation^[88]. This phenomenon has been observed since its early human trials in 2009^[86,89]. Ipilimumab alters the immunological homeostasis, which could lead to the development of autoimmune conditions. It can precipitate an IBD-like disease state and also worsen pre-existing autoimmune disorders like rheumatoid arthritis, psoriasis and lupus^[90,91]. It may even permanently alter the immune system which could give rise to IBD^[92].

Ipilimumab-associated colitis shares many endoscopic and histologic features of classic IBD. The affected individuals have increased CD4 + T-lymphocytes and plasma cells in the gut mucosa, resembling that seen in IBD^[93]. There are upregulated inflammatory pathways with an increased number of inflammatory cells which in turn stimulate other surrounding inflammatory cells causing further release of cytokines and inducing mucosal injury. Increased inflammatory markers or cells in the affected colonic mucosa include granzyme B, FoxP3, and CD8 + lymphocytes^[88]. It commonly presents in the form of an inflammatory colitis, followed by an enteritis^[94]. Severe forms of the disease are susceptible to develop bowel perforation. Extreme precautions should be taken if diagnostic colonoscopy is performed in these patients with severe disease^[95,96].

The principle of treating such inflammatory conditions of the bowel is, like in case of classic IBD, to inhibit immune-mediated injury. Suppressing immunity with the use of steroids is the first-line therapy. Severe cases that do not respond to steroids, have shown to be effectively treated with infliximab, especially those with deep colonic ulcerations^[97,98]. Vedolizumab (anti-integrin molecule) has also shown to induce remission among patients with ipilimumab-induced colitis^[96,99]. With the advent of programmed cell death-1 inhibitors, the use of ipilimumab could be avoided especially due to its strongly associated adverse events and toxicities^[100]. Some experts also suggest the prophylactic use of budesonide along with ipilimumab. In a published double-blinded randomized controlled trial (RCT), prophylactic use of budesonide was most beneficial if the severity of diarrhea was grade 3 or 4, and the authors recommended its use for grade 2 diarrhea or higher^[101]. Discontinuation of the medication followed by steroid therapy seems to be an effective strategy for treating ipilimumab-induced colitis. Once the symptoms resolve it is reasonable to restart therapy, since the recurrence rate of colitis is about 6% and this is independent of the duration of steroid treatment^[102].

The use of ipilimumab among patients with pre-existing IBD could induce a disease flare. As a general rule, it may be best to avoid it among patients who have an existing diagnosis of IBD^[103]. But one could make an argument for treating those patients in long-term disease remission, especially when administered in combination with corticosteroids. In this situation, we recommend to approach it on a case-by-case basis. Combination therapy of nivolumab and ipilimumab is more effective in treating melanoma than ipilimumab alone but it can increase the risk of developing colitis. In a head-to-head analysis of the two treatment options, combination therapy was associated with a higher incidence of adverse events (54%) than ipilimumab monotherapy (20%)^[104]. The most common severe adverse event was colitis, seen in 13% of overall combination treatment group compared to no cases of colitis in ipilimumab monotherapy group. Cases of nivolumab monotherapy-induced colitis have also been reported resembling characteristics like those of UC^[105].

Currently there are no randomized clinical trials in the evaluation of therapies for checkpoint inhibitor induced colitis, although a majority of them are treated with various steroid formulations^[106]. Another approach for its treatment is the use of fecal microbiome transplantation, where stool from healthy donors is transplanted into the gut of individuals suffering from checkpoint inhibitor-induced colitis^[107]. A small case series has demonstrated the benefit in this therapy. This may also suggest the role of fecal microbiome in pathogenic mechanisms of *de novo* IBD^[108].

POST-SURGICAL SECONDARY IBD

Abdominal and pelvic surgeries are commonly performed in IBD patients, especially among those with CD. Studies have shown that bowel altering surgeries can have an effect on the microbiome in the GI tract, creating an environment for IBD remission or flare^[109]. For example, surgical ileocolonic resection in patients with isolated CD resulted in 10-year disease remission in 50% of cases^[110]. But some patients developed postoperative recurrence of bowel inflammation following the same surgery. A murine study showed that ileocolonic resection can alter the microbiota not just in the colon but also in the jejunum, which could precipitate IBD in the large and small bowel^[111]. Animal studies have shown that surgical changes alter the gut microbiome, which can make the commensals virulent and cause anastomotic leaks^[112,113]. These chronic mucosal lesions may represent an IBD-like phenomenon in the post-surgical bowel segments. Individuals with IBD with an abundance of bacterial species like *Bacteroides vulgatus*, *Clostridium perfringens* and *Ruminococcus gnavus* in the gut are at an increased risk for the development of CD of the pouch if they undergo

proctocolectomy with ileal pouch-anal anastomosis (IPAA)^[114].

Post-colectomy enteritis syndrome

Patients with UC that undergo total colectomy or total proctocolectomy can develop a chronic inflammatory state of the small bowel called post-colectomy enteritis syndrome^[115,116]. It is characterized by diffuse chronic enteritis which usually develops several months after the surgery. When suspected, an upper GI endoscopy, ileoscopy *via* stoma, or enteroscopy should be performed^[117]. It appears to be distinct from CD in terms of its non-segmental involvement, superficial mucosal inflammation, absence of fistulas/strictures and the absence of granulomas. This condition can present with severe ulcerations and may even lead to a fatal outcome^[117,118]. This has been speculated to be a form of UC of the small bowel due to its lack of typical features of CD. It is usually treated with immunosuppressive therapy like corticosteroids especially during the initial presentation or a flare, followed by a long-term use of immunosuppressants like azathioprine or infliximab^[117].

Post-colectomy ileal pouchitis

Surgical resection of diseased bowel can be curative among individuals with severe forms of IBD which is unresponsive to medical treatment. Total proctocolectomy with IPAA is considered a definitive treatment for those with UC, since the entire colon with almost complete rectum is removed, leaving no available organ to manifest the disease. CD on the other hand is a segmental disease that can involve any part of the GI tract, hence resection of the diseased segments can be curative in many cases but disease recurrence is not uncommon^[119,120]. Patients with refractory UC or UC with colitis-associated neoplasia require colectomy. Restorative proctocolectomy with IPAA has become the surgical treatment of choice in those who require total colectomy. This standard bowel reconstruction surgery can be associated with pouchitis or CD of the pouch. Pouchitis, a chronic inflammation of the ileal pouch after IPAA is speculated to occur due to fecal stasis, dysbiosis, altered mucosal immunity, and surgery-associated ischemia. It is commonly treated with antibiotics but some cases do not respond to long-term antibiotic therapy and is termed as chronic antibiotic-refractory pouchitis. Therefore, pouchitis represents a disease spectrum ranging from acute antibiotic-responsive phenotype to chronic antibiotic-refractory entity. The latter form of pouchitis resembles classic IBD in clinical, endoscopic, and histologic features, often requiring immunosuppressive therapy, including biologics^[121,122].

In cases that are refractory to medical therapy, fecal diversion, away from the diseased segment of the bowel is an effective modality of treatment^[123]. Experts consider chronic antibiotic-refractory pouchitis as an independent entity of IBD. Another type of pouchitis is called “diversion pouchitis”. Diversion pouchitis responds to short chain fatty acids therapy^[124]. It is important to differentiate this type of inflammation from ischemic changes which are frequently seen near the stoma and is related to the surgical technique.

Fecal diversion with the construction of a stoma can be performed to treat downstream bowel or perianal diseases in IBD. In some cases, fecal diversion may induce *de novo* IBD in an uninvolved segment of the bowel^[125]; *i.e.*, postcolectomy enteritis syndrome as outlined above. This suggests that surgery is a trigger which influences the immune function in the uninvolved segment, leading to the development of inflammation.

Post-colectomy de novo CD of the pouch

Proctocolectomy with IPAA in patients with a preoperative diagnosis of UC has been shown to induce *de novo* CD among 2.7%-13% of patients who undergo the surgery for an initial diagnosis of UC or indeterminate colitis^[126]. The new disease can develop weeks to even years after the surgery. It is believed that the bowel reconstructive surgery for UC creates a CD-friendly environment. After colectomy the GI transit of consumed food is quick, altering the gut microbiome and creating a CD inducing environment. This along with areas of ischemia due to surgical alteration can give rise to *de novo* CD or induce a disease flare^[126]. The development of new disease may occur in those individuals who already have an underlying high-risk genotype for developing IBD and surgery only acts as a trigger that precipitates its phenotypic expression. The role of other environmental factors like peri-operative use of antibiotics and non-steroidal anti-inflammatory drugs, ischemia, obesity, and anxiety related to the surgical intervention may have a significant contributing effect in giving rise to this disease state.

De novo IBD after bariatric surgery

Morbid obesity is a chronic illness which can increase the risk of developing other comorbidities like diabetes, hyperlipidemia, cardiovascular diseases and several types of cancers including colon cancer. Interventions to treat obesity are mainly directed towards lifestyle changes like adopting a low-calorie diet and adequate exercise. Following these interventions can be a challenge to many and a growing number of obese individuals instead choose to undergo bariatric surgery^[127]. There are several types of bariatric procedures performed in the United States, the commonly performed ones are Roux-en-Y gastric bypass (RYGB), sleeve gastrectomy, and laparoscopic gastric band placement^[128,129].

One of the most effective surgeries for morbid obesity is RYGB and it has been shown to induce *de novo* CD^[130,131]. There have been several reported cases of individuals who developed new-onset IBD after undergoing a bariatric surgery^[132-135]. A recently published case series of 44 patients who developed *de novo* IBD after surgery, demonstrated that there seems to be a higher incidence of developing the disease after RYGB^[133]. The majority of cases were females and CD was the most common type of disease ($n = 31$), followed by UC ($n = 12$) and one case of indeterminate colitis. The median time to develop the disease after undergoing surgery was 7 years. Similar findings were observed in another series of 15 patients, suggesting a possible role of bariatric surgery in the development of SIBD^[136].

It is suspected that the alteration in microbiota or nutritional status could have a role in precipitating the disease in these individuals. Similarly, development of celiac disease after undergoing pancreaticoduodenectomy^[137], Billroth II procedure^[138], pyloroplasty^[138] or IPAA^[139] has been reported. This could suggest an immunogenic response to the surgery leading to immune-mediated gluten sensitivity and therefore giving rise to celiac disease. Individuals who undergo bariatric surgery have shown to develop an alteration in their gut microbiota^[140,141]. This change could lead to colonic dysbiosis which plays an important role in pathogenesis of IBD^[15,16]. In addition, there is alteration in the nutritional parameters in these individuals due to artificially induced malabsorption which could play a role in the disease process, for example vitamin D deficiency and hypoglobulinemia have been linked to pathogenesis of IBD^[84,142]. Interestingly, when the surgery is performed in individuals with well controlled IBD, it has shown to be safe and effective, with an acceptable risk of post-operative complications^[143-145]. Our understanding of these mechanisms is limited^[146].

POST-TRANSPLANT SECONDARY IBD

The immune system plays a central role in the pathogenesis of IBD. Hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT) is performed with the use of steroids and immunomodulators. These agents suppress the immunogenic response of the recipients lymphocytes against transplanted human leukocyte antigens, to prevent immune-mediated rejection^[147]. Therefore appropriate cross-matching is carried out prior to transplantation to prevent human leukocyte antigens-mismatch^[148]. The alteration in the immune system with the use of immunosuppressant's or introduction of foreign antigens could lead to a dysregulated immune response and subsequent development of autoimmune disorders. One such autoimmune conditions is IBD that can develop after HSCT or SOT. Another type of transplantation is that of a healthy donor's fecal material, which is used to treat individuals with recurrent *Clostridium difficile* infection (CDI)^[149]. If the donor suffers from IBD then their gut microbiome may carry the microbiota that is proinflammatory and could lead to development of *de novo* IBD in the recipient^[150,151]. Additionally, the alteration in the gut microbiome may induce an exaggerated immune response to the newly introduced bacteria which can precipitate immune-mediated gut inflammation.

Post-hematopoietic stem cell transplant IBD

HSCT is an effective treatment in several hematopoietic disorders including leukemia. Recipients of stem cell therapy are immunosuppressed and are at risk for developing various GI infections, like infectious enterocolitis. Umbilical cord blood of newborns is a good source of harvesting stem cells which are used for HSCT^[152]. The recipients can develop a unique type of colitis, which is clinically and histologically distinct from the typical infectious colitis or colitis associated with graft-versus-host disease^[153,154]. This condition is termed as "cord colitis" and the affected individuals usually present with non-bloody diarrhea several months after the transplantation^[153]. Colonic biopsies demonstrate chronic active colitis with non-caseating granulomas and the disease can

involve the upper or lower GI tract, a pattern resembling that of CD^[154,155]. They also tend to have high loads of the bacteria *Bradyrhizobium enterica* in their gut. Some speculate that the alteration in gut microbiome has a role in the pathogenesis of this disease^[156]. This is also supported by the fact that antibiotic therapy is effective in the treatment. Cord colitis could be a variant of IBD which develops in the setting of altered immunity due to the use of cord blood stem cells or the use of immunomodulators.

Post-solid organ transplant IBD

Individuals who undergo SOT are immunosuppressed during the peri-transplant and post-transplantation periods. There is increased risk of developing diarrhea after transplantation, commonly due to infections or due to side effects of medications, but there are other conditions that could cause diarrhea in these patients. Two such conditions are the onset of an IBD flare in those with a pre-existing diagnosis of IBD or the development of *de novo* IBD^[157]. This phenomenon is more common after orthotopic liver transplantation than other SOT, like kidney or heart^[158]. IBD in general and UC in particular are associated with concomitant PSC, for which the mainstay of treatment is OLT. This is probably the reason for encountering higher number of OLT than SOT of other organs in the IBD population^[159,160]. The incidence risk of developing *de novo* IBD in SOT recipients versus general population is 206 and 20 respectively per 100000 person-years^[161]. In a study of post-renal transplant recipients, it was found that the incidence risk of developing IBD was twice than that for general population^[162]. In a retrospective chart review of 6800 liver and/or kidney transplant recipients that received some form of immunosuppression, it was found that 14 individuals developed *de novo* IBD^[161]. Post-OLT patients who develop *de novo* IBD had a tendency to have underlying PSC or develop PSC in the future^[160,163].

The etiopathogenesis of *de novo* IBD after SOT is likely related to the use of immunosuppressants like steroids, immunomodulators, and anti-thymocyte globulin; that alter the “immune thermostat” leading to a dysregulated immune response to gut microbiome^[157]. This in turn can lead to development of *de novo* IBD among at risk individuals. This is a paradoxical response to immunosuppression, a key principle in treating IBD with agents like azathioprine and 6-MP. Details regarding effects of various immunomodulators in precipitating IBD have been described in the previous section of this review. In a recently published retrospective study of 373 patients suffering from PSC, with or without concurrent IBD, the 10-year cumulative risk of developing a disease flare or *de novo* IBD was about 25%^[42]. These risks were higher with the use of MMF and lower with azathioprine in the post-operative period. The use of azathioprine after SOT seemed to be protective against the development of IBD flare or *de novo* IBD^[29,42].

Interestingly, there is evidence suggesting that immunosuppression with organ transplantation may be beneficial in patients with IBD. In a study of 41 IBD patients that underwent OLT the rate of clinical remission was higher than 42 IBD patients who did not undergo OLT (54% *vs* 33%, $P = 0.03$)^[164]. Transplant recipients who received MMF had better outcomes than those with other immunosuppressants. This variable response to immunotherapy by different individuals illustrates the complex nature of the immunological processes at play where alteration in immunity causes a disease flare in some while in others it could be protective against flaring. One could argue that the transplant recipients were monitored closely and were more compliant with medications, therefore had lesser incidence of disease flare.

Fecal microbiota transplantation-associated IBD

Gut microbiome consists of trillions of organisms which include bacteria, fungi and viruses. In patients with IBD there is a state of gut dysbiosis which plays a role in its pathogenesis^[150,151,165]. Individuals with recurrent CDI have a significant alteration of their colonic microbiota leading to gut dysbiosis, which can be successfully treated with fecal microbiome transplantation (FMT) from healthy donors^[149]. The fecal material is introduced *via* upper delivery methods (oral capsules or naso-gastric/jejunal tube) or lower delivery methods (colonoscopy or enema). Among individuals with IBD who develop CDI, FMT has shown to be successful in treating 87%-91% of cases after a single transplantation^[166,167]. Interestingly, this rate of success is slightly lower when compared to individuals without IBD^[168]. Since gut dysbiosis is seen in IBD as well, investigators have utilized FMT for treating IBD flares with good response, especially in case of UC^[169,170]. There are few reported cases of CD flare that responded successfully to FMT^[171,172]. It may also have a role among patients with active disease that is not responding to biologic therapy^[172]. Fecal transplantation has also shown to modestly improve disease severity in case of chronic pouchitis^[173].

It is interesting to note that individuals treated with FMT for CDI appear to have an alteration of gut microbiome mirroring the donor's microbiome and with a higher microbial biodiversity^[174]. But in patients with IBD the microbiome after FMT resists this change and rather tends to retain the pre-transplant type of gut microbiome. This may suggest that the microbiome by itself may not be an influential pathogenic mechanism in IBD but rather a source to stimulate the immune system that drives the disease process and which in turn influences the gut microbiome. This may also explain the lack of consistent response of FMT in treating IBD. A meta-analysis of nine cohort studies, eight case studies and one RCT showed that FMT appears to be safe in IBD but its efficacy was not consistent^[169]. In a more recent meta-analysis of four RCT's comparing FMT versus placebo for the treatment of active UC, showed that clinical remission was achieved in 28% of patients in FMT group when compared to only 9% in placebo group^[170]. Rates of clinical response were 49% and 28% in the FMT and placebo groups, respectively. Well-designed RCT's are needed to further study the role of FMT in the treatment of IBD^[175].

Use of FMT is not a completely safe procedure and is associated with few adverse events, especially infections and in rare cases death^[176,177]. There have been multiple reported cases of individuals with IBD and CDI who developed an IBD flare after treatment with FMT^[176,178,179]. Studies have shown that the rate of developing a disease flare after FMT is about 15%-25%, higher among those who were transplanted *via* lower delivery method than peroral method^[168,180]. Based on these observations, we can speculate that FMT could also induce development of *de novo* IBD among individuals who carry the preferential genotype for the disease. This may be more likely if the donors themselves have active IBD. As a part of screening process for donors, several experts that FMT continue to exclude those who have IBD, even though we have not had any direct evidence that shows that such donors with IBD could cause adverse effects in the recipient^[181-183]. Perhaps the alteration of gut microbiome may be more harmful in certain individuals and further studies are needed to recognize the factors that influence the development of FMT-induced IBD flare up.

CONCLUSION

IBD have a complex pathogenesis which consists of an interaction between immune system, gut microbiome and genetic factors. These three components can be influenced by several environmental factors which could tip the balance towards an immune mediated proinflammatory state in the gut and in certain extra-intestinal tissues. In a normal state there is immune tolerance towards host microbiome. External influences can alter this balance by inducing a hyper-immune response, leading to mucosal injury of the GI tract. This type of *de novo* IBD due to specific external causes is termed as secondary inflammatory bowel disease (SIBD). These external factors have been categorized into three main groups: Drugs, surgery and organ/fecal transplantation. Drugs that can influence immunity and potentially alter it, have been implicated with the development of drug-induced SIBD; these include immunomodulators, biologics, interferons, immune stimulators, and immune checkpoint inhibitors. Bowel altering surgery can influence the microbiome and lead to malabsorption, especially in case of bowel resection and bariatric surgery. Surgeries that are used to treat one type of disease, like UC with proctocolectomy followed by ileal pouch-anal anastomosis, have shown to precipitate new forms of diseases like chronic pouchitis or CD of the pouch. This type of *de novo* chronic gut inflammation after surgery is termed as post-surgical SIBD. The role of immunosuppressants in organ transplant recipients has been key in preventing immune-mediated rejection. These immunosuppressants can paradoxically induce an autoimmune mediated gut inflammation. The donor's foreign organs or stem cells could also induce an immune reaction causing immune mediated tissue injury. Both these factors can lead to post-solid organ/stem cell transplant related. The interaction between microbiota and host immune system is complex process, and factors altering bowel anatomy and gut homeostasis may reset host's immune thermostat, triggering the development of IBD or SIBD.

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Clinical considerations in the management of non-alcoholic steatohepatitis cirrhosis pre- and post-transplant: A multi-system challenge

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Abstract

Non-alcoholic steatohepatitis (NASH) is the most common chronic liver disease worldwide, and the fastest growing indication for liver transplantation in the United States. NASH is now the leading etiology for liver transplantation in women, the second leading indication for men, and the most common cause amongst recipients aged 65 years and older. Patients with end-stage liver disease related to NASH represent a unique and challenging patient population due the high incidence of associated comorbid diseases, including obesity, type 2 diabetes (T2D), and hypertension. These challenges manifest in the pre-liver transplantation period with increased waitlist times and waitlist mortality. Furthermore, these patients carry considerable risk of morbidity and mortality both before after liver transplantation, with high rates of T2D, cardiovascular disease, chronic kidney disease, poor nutrition, and disease recurrence. Successful transplantation for these patients requires identification and management of their comorbidities in the face of liver failure. Multidisciplinary evaluations include a thorough pre-transplant workup with a complete cardiac evaluation, control of diabetes, nutritional support, and even, potentially, consultation with a bariatric surgeon. This article provides a comprehensive review of the conditions and challenges facing patients with NASH cirrhosis undergoing liver transplantation and provides recommendations for evaluation and management to optimize them before liver transplantation to produce successful outcomes.

Key words: Liver transplantation; Non-alcoholic fatty liver disease; Obesity; Metabolic syndrome

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Core tip: Non-alcoholic steatohepatitis (NASH) is the most common chronic liver disease worldwide, and the fastest growing indication for liver transplantation (LT) in the United States. Patients with NASH represent a unique and challenging population due to the high incidence of associated conditions (*i.e.* obesity, diabetes, and hypertension), which carry considerable risk of morbidity and mortality before and after LT due to cardiovascular disease and kidney disease. This article provides a comprehensive review of the conditions and challenges facing patients with NASH and provides recommendations for evaluation and management to optimize them before LT.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a global epidemic and the most common cause of chronic liver disease worldwide^[1]. NAFLD represents a spectrum of liver disease, starting with simple steatosis (NAFL) and progressing to non-alcoholic steatohepatitis (NASH) with inflammation and cellular injury in addition to fat accumulation^[2]. Livers affected by NASH may ultimately develop fibrosis and progress to cirrhosis and liver failure requiring liver transplantation (LT)^[3]. While chronic infection with hepatitis C virus (HCV) has long-been the leading indication for liver transplantation, the recent advent of direct antiviral agents has resulted in increased rates of disease resolution and decreased the need for LT^[4-6]. Simultaneously, the increasing prevalence of obesity throughout the world has led to an increased incidence of NASH and NASH-related cirrhosis^[1]. Importantly, NASH is now the leading indication for LT in women, the second leading indication for men, and the most common non-malignant indication amongst recipients aged 65 years and older^[7,8].

NASH cirrhosis represents a growing challenge in transplantation with no effective treatment. Strongly associated with the metabolic syndrome, patients with NASH often have the associated comorbidities of obesity, type 2 diabetes (T2D), cardiovascular disease, and chronic kidney disease, amongst others^[9-12]. This constellation of diseases, along with end-stage liver disease (ESLD), makes treating patients with NASH cirrhosis a challenging clinical endeavor. Furthermore, these conditions increase the risk of transplantation and may complicate post-LT immunosuppression and care.

To address this unique clinical challenge, here we present a comprehensive review article in which we discuss the difficulties in managing patients with NASH before and after LT, with consideration given to the interplay of disease physiologies and potential treatments where available.

PRE-TRANSPLANT CONSIDERATIONS FOR PATIENTS WITH NASH

NASH is the fastest rising cause of ESLD amongst registrants on LT waitlists in the United States, with a 170% increase from 2004 to 2013^[13]. The number of LT performed for NAFLD increased fourfold between 2002 and 2012^[6]. During nearly the same time, the mean age of all LT recipients increased, and the increase in age amongst HCV-negative patients was associated with an increase in NASH cirrhosis^[14]. NASH has become the most common indication for LT amongst patients ≥ 65 years old^[8]. Recently, Parikh *et al*^[15], using national data to model the rise of NASH in LT in the United States, predicted a 55.4% increase in NASH-related waitlist additions by 2030. In concert with decreasing prevalence of HCV^[16], NASH will likely become the most common indication for both waitlisting and receipt of LT in the next 15 years^[15,17]. In addition to aging, NASH has a predilection for the female gender. Our group recently

showed that NASH is the leading indication for LT waitlist registration and transplantation for women^[7].

NASH and obesity

NASH patients are a unique and complex population, with multiple comorbidities complicating their underlying liver disease (Figure 1). Obesity is a growing epidemic in the United States, with an estimated 38% of adults having a body mass index (BMI) > 30 kg/m²^[18]. Obesity alone has been a point of contention in LT^[19]. In the pre-model for end-stage liver disease (MELD) era, Nair *et al*^[20] considered morbid obesity (BMI ≥40 kg/m²) an independent predictor of mortality in LT recipients. In contrast, Leonard *et al*^[21] evaluated LT outcomes by recipient BMI after removing ascites and found no difference in survival. Nonetheless, obesity has been associated with increased rates of early graft dysfunction, longer hospital stays, and increased rates of infection in the United States and the United Kingdom^[20,22,23]. In the pre-LT setting, Segev *et al*^[24] found that obese patients were more likely to be turned down for organ offers and to receive fewer MELD exception points than were leaner individuals. There is a trend towards worse outcomes when BMI is > 40 kg/m² and with concomitant diabetes^[25,26]. Overall, the International Liver Transplantation Consensus Statement on ESLD due to NASH does not recommend against LT on the basis of obesity alone but supports careful patient selection in the presence of comorbidities^[27].

NASH is the result of progression from NAFL and is often considered the hepatic manifestation of the metabolic syndrome^[2]. The syndrome has been defined in a joint publication of the International Diabetes Foundation and the National Heart, Lung, and Blood Institute in the United States (Table 1). In addition to being associated with older aged and female patients, NASH is also commonly seen with obesity, hypertension, diabetes, renal disease and cardiovascular disease^[28].

Insulin resistance, metabolic syndrome and NASH

Insulin resistance likely is the primary pathogenetic factor that ties metabolic syndrome and NAFLD/NASH together. In the liver, elevated serum glucose and insulin values increase the activity of carbohydrate response element binding protein and sterol regulatory-element binding protein 1c, which leads to impaired metabolism of liver lipid, increased lipid deposition, and further inhibition of insulin signaling within the liver^[29-31]. Hepatic insulin resistance and steatosis may be the “first hit” in the development of NAFLD, sensitizing the liver to “second hits,” which lead to the development of inflammation, fibrosis, and necrosis that are characteristics of NASH^[32,33]. The second hits are multifactorial – inflammatory cytokines, adipokines, mitochondrial dysfunction, oxidative stress, breakdown of the gut mucosal barrier with endotoxemia, and activation of Kupffer cells and hepatic stellate cells^[34-38].

Not surprisingly, diabetes is common amongst LT candidates with NASH. The incidence of diabetes amongst patients awaiting LT with NASH is more than 2-fold higher than any other causes, ranging from 46%-55%^[13,39]. Hoehn *et al*^[40] reported that NASH was the most common cause of ESLD amongst patients undergoing LT with diabetes. Furthermore, the severity of liver disease in NAFLD/NASH may be related to T2D. In a 2006 study examining the association between NAFLD and diabetes, 71% of patients with biopsy- proven NASH had diabetes, whereas only 46% of patients with simple steatosis had the disorder^[41]. Importantly, pre-LT diabetes is associated with early postoperative complications, such as infection and adverse cardiovascular events^[42].

Hypertension is another component of the metabolic syndrome seen commonly in LT candidates with NASH^[43]. In an evaluation of listed patients, hypertension was present in 46% of those with NASH compared with 28% of those with HCV^[39]. An independent association between NAFLD/NASH and hypertension has been reported^[44,45]. While hypertension is not prevalent amongst individuals awaiting LT, pathogenetic mechanisms associated with arteriolar hypertension may contribute to the increased incidence of renal dysfunction and cardiovascular risk in patients with NASH^[46].

Renal dysfunction with NASH

Patients with NASH commonly have multiple risk factors for chronic kidney disease (CKD). CKD, defined as decreased estimated glomerular filtration rate (eGFR) and/or overt proteinuria and/or abnormal albuminuria, is common in patients with NAFLD and NASH, with a prevalence of 20%-55%^[47]. While the development of CKD in these patients is likely related in part to the end-organ effects of diabetes, hypertension, and insulin resistance, distinct pathogenetic mechanisms due to NASH per se are

Table 1 Metabolic syndrome criteria

Metabolic syndrome criteria	
Characteristic	Description
Waist circumference	≥ 88 cm in females
	≥ 102 cm in males
Triglycerides	≥ 150 mg/dL
HDL	On drug treatment for elevated triglycerides
	≤ 40 mg/dL for men
	≤ 50 mg/dL for women
Hypertension	On drug treatment for low HDL
	Systolic blood pressure ≥ 130 mmHg
	Diastolic blood pressure ≥ 85 mmHg
Diabetes	On anti-hypertensive drug treatment for history of hypertension
	Elevated fasting glucose ≥ 100 mg/dL
	On drug treatment for elevated glucose

Patients must exhibit 3 of the 5 components to have the diagnosis with metabolic syndrome. Based on consensus statement from International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity^[182]. HDL: High-density lipoprotein.

possible^[48-51], as NAFLD and NASH have been independently associated with both the prevalence and incident of CKD^[52-54], where the risk of developing CKD has hazard ratios (HR) of 1.49-1.85.

The severity of CKD has been related also to the severity of liver disease. Yasui *et al*^[55] examined 174 Japanese patients with NAFLD and found a higher rate of CKD with NASH than with simple steatosis (21% *vs* 6%, *P* = 0.007). Another study evaluated 80 patients with biopsy-proven NASH and found that eGFR decreased with increasing degrees of hepatic fibrosis^[56]. Musso *et al*^[57] presented the most comprehensive evaluation of NAFLD/NASH and CKD in a meta-analysis, which included 63902 patients and 33 studies; that study found both an independent association between NAFLD and CKD in both diabetic and non-diabetic patients, and higher prevalence and incidence of CKD with NASH than with simple steatosis.

CKD may affect all patients with NAFLD and NASH, but it is especially problematic for patients awaiting LT. Park *et al*^[43] evaluated waitlisted patients and found higher serum creatinine values and prothrombin times in patients with NASH than in those with other causes of ESLD and the same MELD score; this observation was confirmed by Wong *et al*^[13], who found a lower eGFR amongst waitlisted patients with NASH than amongst those with other causes of ESLD. The presence of renal dysfunction and CKD prior to LT is a risk factor for post-LT CKD and is associated with worse graft and patient survival^[58-60]. Fussner *et al*^[58] reported that NASH and female gender were independently associated with CKD at 1 year after LT. Houlihan *et al*^[61] reported similarly higher rates of stage III CKD in patients with NASH than in those with liver disease of other causes (31.2% *vs* 8.3%, *P* < 0.001) at 2 years after LT; however, they found no difference in 1-year or 5-year patient or graft survival. Importantly, however, patients with NASH are more likely than those with ESLD from other etiologies to require renal replacement therapy prior to transplantation, which carries a 150% increased risk of mortality before transplantation^[62,63].

Simultaneous liver and kidney transplantation (SLKT) is an option for patients with NASH cirrhosis and CKD. NASH is the fastest rising indication for SLKT in the United States, increasing from 6.3% of SLKT in 2002 to 19.2% in 2011^[64]. In a comparison with patients undergoing SLKT for alcoholic cirrhosis, NASH, and HCV, Singal *et al*^[65] found similar 5-year liver allograft survival but significantly worse renal allograft survival and a 1.5-fold increased risk of renal graft loss. Molnar *et al*^[66] compared pre-LT eGFR and post-LT renal recovery in 4,088 NASH LT recipients from the United Network for Organ Sharing database. Over a median follow-up of 5 years, NASH patients with preserved renal function had a lower risk of death than did those with eGFR < 30 mL/min; however, similar rates of death and graft loss were seen for

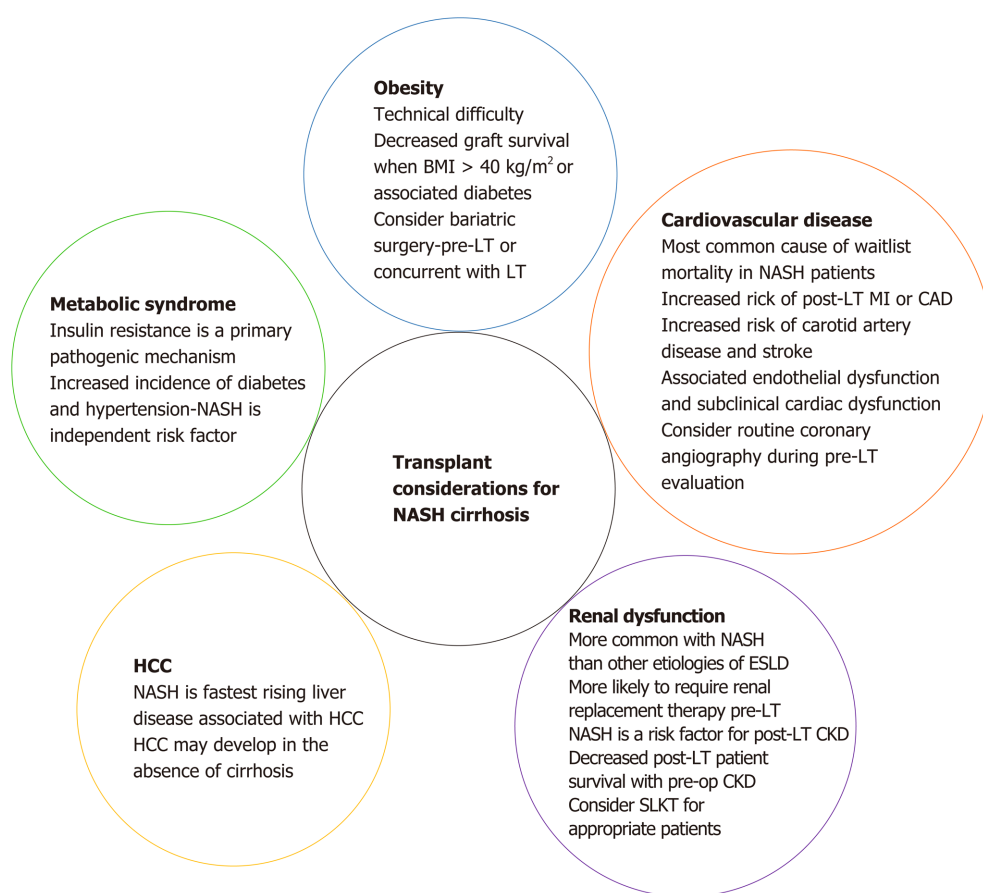


Figure 1 Optimizing patients with non-alcoholic steatohepatitis cirrhosis for liver transplantation. Patients with non-alcoholic steatohepatitis cirrhosis represent a unique and challenging population. Comorbid conditions which may complicate pre- and post-transplant care are presented along with considerations for optimization. BMI: Body mass index; LT: Liver transplantation; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; ESLD: End-stage liver disease; CKD: Chronic kidney disease; SLKT: Simultaneous liver and kidney transplantation.

NASH patients with SLKT and as those with reduced renal function^[66].

Cardiovascular disease and NASH

Increasing literature supports an increased risk of cardiovascular events in patients with NASH. Cardiovascular disease (CVD) is a leading cause of mortality in LT patients, accounting for 19%-42% of non-graft-related mortality^[67,68]. In LT patients, CVD is associated with typical risk factors: Diabetes, hypertension and renal dysfunction^[67]. Additionally, recent research supports NAFLD and NASH as independent risk factors for the development of CVD^[69,70].

The pathogenetic mechanisms for CVD in patients with NAFLD are multifactorial and incompletely understood. In addition to the typical risk factors—hyperlipidemia, hypertension and impaired glucose tolerance—characteristics unique to NAFLD, have been found independently associated with endothelial dysfunction^[71-73]. Arterial stiffness may play a role and has also been associated with NAFLD^[74-78]. Endothelial dysfunction is a separate but inter-related mechanism that is common with atherosclerosis and is regulated by multiple mechanisms^[79]. As both a result and mediator of arterial changes, NAFLD has been associated with increased expression of biomarkers of endothelial dysfunction, such as sICAM-1 and plasminogen activator inhibitor-1 (PAI-1)^[80,81]. PAI-1 is not just a marker of endothelial dysfunction, but is also prothrombotic and associated with increased risk of myocardial infarction^[82,83]. Changes in cardiac function also are present in patients with NAFLD: Kim *et al*^[78] showed that NAFLD was independently associated with left ventricular diastolic dysfunction. Insulin resistance is a primary contributor to cardiac dysfunction, being associated with myocyte growth, interstitial fibrosis, sodium retention and changes in sympathetic nervous system activation^[84,85].

Unfortunately, much of the cardiac dysfunction in NAFLD is subclinical and difficult to diagnose. NAFLD has been associated with decreased myocardial

perfusion reserve, which may make patients with NAFLD prone to subendocardial ischemia in the presence of hemodynamic compromise^[86]. LT screening guidelines recommend that dobutamine stress echocardiography be performed, and, if abnormal, be followed with coronary angiography^[87,88]. In a study of patients with NAFLD undergoing LT evaluation, 37% did not reach target heart rate during stress echocardiography^[89]. Tests of cardiac function in NAFLD patients may not reveal the severity of disease: A meta-analysis of cardiac stress test results during LT evaluation revealed a pooled sensitivity of 21-28% and specificity of 82-91% for coronary artery disease^[90]. Dobutamine stress echocardiography has poor predictive value for post-operative cardiovascular events, with a reported positive predictive value of 6.7% and negative predictive value of 83.5%^[91]. Prolonged QT segment may be a marker for cardiac dysfunction in NAFLD, and changes in cardiac morphology may lead to the development of atrial fibrillation, which has been independently associated with NAFLD^[92,93]. Importantly, atrial fibrillation is a risk factor for both intra-operative and post-operative cardiac events in LT^[94].

An early study by Kadayifci *et al*^[95] reported an increased prevalence of coronary artery disease associated with NASH-related cirrhosis compared other causes of liver disease (21.6% *vs* 5%, $P = 0.005$ respectively). Similarly, Patel *et al*^[96] found an increased risk of severe coronary artery stenosis, defined as stenosis > 70% on angiography, in patients with non-alcohol related ESLD. Carotid artery disease also is increased in patients with NASH^[97]. Carotid intima-media thickness, a marker of atherosclerosis, is associated with increased risk for myocardial infarction, cerebrovascular accidents and peripheral vascular disease^[98]. Two studies have found increased carotid intima-media thickness in patients with NAFLD^[99,100].

An increased risk of CVD events with NAFLD before and after LT has been reported^[101-105]. CVD has been found the reason for waitlist mortality more often in patients with NASH than in those with other kinds of ESLD^[13], and Vanwagner *et al*^[89] have reported that patients who underwent LT for NASH were more likely to die of a cardiovascular event within 1 year post-LT than were those who had LT for alcoholic cirrhosis (adjusted OR = 4.12, 95%CI: 1.91-8.90). The same group later reported a higher incidence of sudden cardiac death or acute heart failure in patients transplanted for NASH than in those transplanted for other causes of ESLD^[106]. A systematic review and meta-analysis comparing patients with LT for NASH with those without NASH supported these findings, showing that the recipients who had NASH had higher rates of death due to CVD^[107].

Bariatric surgery and NASH

Patients with NAFLD and obesity should pursue exercise and nutrition counseling. However, dieting, exercise, and behavioral therapy are poorly tolerated by those patients who have severe liver disease^[108]. As obesity in patients with NASH cirrhosis might prohibit LT, bariatric surgery has been proposed as an option^[109]. Weight loss surgery can reduce the burden of comorbidities in patients with NASH, resulting in weight loss and improvement in T2D, hypertension, and insulin resistance^[110,111]. Bariatric surgery in this population should be sleeve gastrectomy rather than gastric banding or gastric bypass, as the latter procedures might make the anatomy difficult for LT. Sleeve gastrectomy results in excellent weight loss and additionally has the benefit of not being a malabsorptive procedure, which may otherwise impact absorption of immunosuppressive medications post-LT.

Optimal timing of bariatric surgery for patients with NASH has not been determined; various groups have reported successful outcomes when the surgery is performed prior to, concurrent with, or after LT^[112]. Shimizu *et al*^[113] performed bariatric surgery in 23 patients with cirrhosis (22 with Child's A cirrhosis); 14 patients underwent laparoscopic roux-en-y gastric bypass and 8 underwent laparoscopic sleeve gastrectomy. Overall, mean weight loss was approximately 35 kg, diabetes resolved or improved in 87%, and hypertension resolved or improved in 69%. The rates of complication were similar between the 2 procedures (28.6% for bypass *vs* 37.5% for sleeve gastrectomy; $P > 0.05$). A case series from France of 109 patients with NASH who underwent bariatric surgery had similar improvement in BMI, but, more important, had improvement in features of NASH: Less hepatocellular ballooning in 84.2% and reduction in lobular inflammation in 67.1%^[114]. However, this study included mostly NASH patients without cirrhosis.

The presence of decompensated cirrhosis, however, may prohibit elective weight-loss surgery. For these patients, bariatric surgery at time of transplantation may be an option. Heimbach *et al*^[115] published one of the first case series with this approach, on patients listed for LT with BMI > 35 kg/m². Seven patients were unsuccessful in pre-LT weight loss and ultimately underwent simultaneous LT and sleeve gastrectomy,

with a median MELD score of 32 and BMI at transplantation of 48 kg/m². Post-LT, all 7 patients had resolution of diabetes and hypertension and achieved a BMI below 35 kg/m². Only 1 patient had a complication related to the bariatric surgery procedure, a leak at the gastric staple line. Since this initial report, a few more small case series have been published with similar findings^[116,117]. Zamora-Valdes *et al*^[118] recently updated the long-term results from the initial study, reporting that patients who underwent combined transplantation and sleeve gastrectomy maintained weight loss and had a lower incidence of diabetes, hypertension, and hepatic steatosis at 3 years after LT than did those who had pre-LT weight loss without bariatric surgery. Bariatric surgery after transplantation remains an option for obese patients; however, this approach is more technically complicated because of adhesions and increased risk of immunosuppression-related complications^[119-121].

Other issues for LT in patients with NASH

Over the past 5 years, the nutritional status of patients with ESLD has become increasingly recognized as an important factor in outcomes. Despite increased weight and BMI, many obese individuals are nutritionally depleted, with muscle wasting and fatty muscle infiltration, which can lead to sarcopenic obesity^[122-124]. Carey *et al*^[125] performed a multicenter study to better define sarcopenia in LT, finding that skeletal mass index was independently associated with waitlist mortality and identifying cutoffs to define sarcopenia (< 50 cm²/m² for men and < 39 cm²/m² for women). Carias *et al*^[126] identified NASH as an independent predictor of sarcopenic obesity.

Portal vein thrombosis (PVT) is a common complication of chronic liver disease and is a risk factor for graft loss in patients with cirrhosis^[127-131]. Patients with NAFLD have been found at higher risk for venous thromboembolism, such as pulmonary embolus or deep vein thrombosis^[132]. Patients with NASH cirrhosis are at increased risk also for pre-LT PVT^[133]. Stine *et al*^[134] reported that, amongst patients with NASH, those who are older than 60 years and have a BMI > 30 kg/m², hypertension and diabetes, have an even higher risk of pre-LT PVT. Agbim *et al*^[135] recently reported a 37% increased risk of graft loss and a 31% increased risk of death amongst patients who underwent LT for NASH cirrhosis with pre-LT PVT compared to those without PVT.

Hepatocellular carcinoma with NASH

In addition to cirrhosis, NASH is associated also with the development of hepatocellular carcinoma (HCC), with an estimated incidence of 2.6% per year^[136]. NASH is the fastest rising cause of HCC in LT^[137]. Data from two North American centers reveal that the proportion of LT for NASH-related HCC rose from 4% to 9% between 2004 and 2014^[138]. In a separate evaluation of data on Scientific Registry of Transplant Recipients, Younossi *et al*^[137] found that the proportion of LT candidates who had NASH-related HCC increased 7.7-fold between 2002 and 2016 (2.1% to 16.2%, $P < 0.0001$), while the proportions of HCC related to HCV and alcohol-related liver disease remained stable. Moreover, up to 38% of patients with NASH and NAFLD may develop HCC, even in the absence of cirrhosis^[139,140]. Survival outcomes for patients transplanted with HCC due to NASH do not seem to differ from outcomes with transplantation for other causes of HCC^[137].

Like other comorbidities associated with NASH, insulin resistance, oxidative stress, and an inflammatory environment contribute to the development of HCC^[141,142]. Furthermore, over 28000 somatic mutations have been identified in HCC^[143]. Grohmann *et al*^[144] have described an independent mechanism in which obesity contributes to development of HCC through activation of signal transducer and activators of transcription (STAT)-1 and STAT-3 signaling. Together, STAT-1 and STAT-3 create a pro-inflammatory environment and drive oncogenesis, respectively^[145,146]. Undoubtedly, as obesity and NAFLD become more prevalent, more mechanisms contributing to the pathogenesis of NASH and HCC will be identified.

NASH and the waitlist

The combined effects of comorbidities yield a NASH population with complex systemic diseases. Unfortunately, this complexity compounds patients' pre-transplant management. O'Leary *et al*^[39] reported that NASH patients presenting for LT evaluation were more likely than others to be denied listing because of their comorbidities (72%). They also were more likely than patients with HCV to be removed from listing due to death or being "too ill" for transplantation (22% vs 16%, $P = 0.006$) and were less likely to receive a transplant (27% vs 46%, $P < 0.001$). Notably, when patients had MELD scores > 15, there was no difference in rate of transplantation, removal from waitlist, or progression of MELD score. Wong *et al*^[13]

found that NASH patients, compared with patients with alcoholic liver disease, had a lower rate of transplantation and increased mortality rate at 90-days from listing, but this discrepancy disappeared at 1-year after listing. More recently, in an examination of patients on the United Network for Organ Sharing waitlist from 2002 to 2016, Thuluvath *et al*^[147] found that NASH patients also had a slightly higher unadjusted incidence of death or deterioration (29%) than did those with alcoholic liver disease (28%, $P > 0.05$); however, multivariable analysis showed that much of the difference could be attributed to factors associated with NASH (*i.e.*, older age and diabetes) and not to NASH independently. In light of these findings, no scoring system for pre-transplant mortality has been developed specifically for patients with NASH cirrhosis. To date, the MELD score is the most validated predictor of pre-transplant mortality regardless of etiology. Because of their older age, obesity, and multiple comorbidities, waitlisted NASH patients face numerous challenges: As waitlists for transplantation grow longer and the median MELD score at transplantation continues to rise, patients with NASH are at an ever-increasing risk for poor outcomes before reaching LT.

Older age and multiple comorbidities make patients with NASH who are undergoing LT evaluation a highly complex population. Proper pre-transplant evaluation includes a thorough assessment for diabetes, hypertension, renal dysfunction, and cardiovascular risk factors. **Table 2** highlights our recommendations for each of these conditions. As with all patients undergoing LT, optimization of medical comorbidities is a necessity to achieve successful outcomes. We also recommend that obese patients undergo consultation with a nutritionist, an exercise therapist, and, if felt indicated, a bariatric surgeon. For obese patients with diabetes and/or hypertension who are not candidates for elective bariatric surgery, we recommend consideration of performing sleeve gastrectomy at the time of transplantation, but this consideration deserves scrutiny.

OUTCOMES FOR LT IN NASH RECIPIENTS

Overall survival

Prognosis after LT for NASH is generally acceptable. A recent large-volume ten-year review from the Scientific Registry of Transplant Recipients (SRTR) found 1- and 3-year patient survival rates of 84% and 78% after LT for NASH compared with 87% and 78% for other indications^[148]. No significant difference in 5-year graft loss or mortality was observed, suggesting that NASH itself is not an independent risk factor for mortality. In a meta-analysis of 16 studies on post-LT survival with NASH, most studies found no significant survival difference was found between NASH and other etiologies of liver disease^[149]. Another study documented superior survival in NASH patients compared with LT recipients for other causes for transplantation, such as HCC, hepatitis C or alcoholic liver disease^[150].

Mortality after LT for NASH patients is most common within the first few years after transplantation, with cardiovascular events being the primary cause^[151-153]. Furthermore, the incidence of mortality from cardiovascular causes is 15% higher in NASH patients in the first year, but this difference is not sustained beyond the first postoperative year. Kennedy *et al*^[154] reported long-term mortality in NASH patients after LT is primarily associated with malignancy (recurrent HCC and extrahepatic malignancy), cardiovascular complications, and infectious complications. A study by Bhati *et al*^[155] also identified pre-transplant obesity (BMI > 30 kg/m²) and age of 60 years at the time of transplantation as predictors of post-LT mortality.

Complications

Despite lack of non-inferior survival data, the overall incidence of morbidity after LT appears to be higher for NASH recipients than others^[156]. Metabolic syndrome develops in up to 50% of patients after LT for NASH; however, no significant difference between NASH and other etiologies of liver disease has been shown^[149]. Nonetheless, it is postulated that LT recipients with NASH have a predisposing metabolic milieu that persists despite transplantation, and it may be further modulated by steroid-based immunosuppressive regimens. A strong correlation between metabolic syndrome and insulin resistance has been suggested, but this relationship has been poorly studied in the LT population.

Pre-existing diabetes is often cited as the leading cause of post-transplant morbidity, owing to impaired neutrophil function and increased susceptibility to infection with post-transplant hyperglycemia^[156-158]. New onset diabetes after LT is associated with pre-transplant glucose intolerance, obesity, and family history, but the toxic effects of

Table 2 Recommendations for pre-transplant evaluation in patients with non-alcoholic steatohepatitis cirrhosis

Evaluation and therapy for liver transplant candidates with non-alcoholic steatohepatitis	
Hypertension	Target blood pressure 130/80 Initiate anti-hypertensive medical therapy
Diabetes	Blood glucose control Monitor insulin resistance Hemoglobin A1c optimization
Hyperlipidemia	Initiate statin therapy as appropriate
Renal dysfunction	Renal ultrasound Measure GFR by quantitative method Consider simultaneous liver/kidney transplantation
Cardiovascular disease	Identify cardiovascular risk factors—hypertension, diabetes, hyperlipidemia Comprehensive cardiac evaluation to include EKG, DSE Strong consideration for coronary angiography, in addition to OR in place of DSE Carotid artery duplex
Obesity	Consultation with nutritionist or dietician and exercise therapist Consider consultation with bariatric surgeon Consider pre-transplant or simultaneous LT + bariatric surgery if fail weight loss strategies with concurrent comorbid conditions

GFR: Glomerular filtration rate; EKG: Electrocardiography; DSE: Dobutamine stress echocardiogram; LT: Liver transplantation.

immunosuppressants on pancreatic B cells (particularly by calcineurin inhibitors) may play a role in its etiology^[159,160]. Likewise, new onset obesity post-transplant occurs more often in NASH patients than in those with liver disease of other causes, and is closely linked to post-LT diabetes and *de novo* NASH^[161,162]. In a study examining BMI change after LT, 22% of 320 recipients who were not obese pre-transplant became obese within 2 years after transplantation^[163].

Several studies have reported a higher frequency of hypertension and dyslipidemia in post-LT patients with NASH than with other liver diseases and is often related to immunosuppression^[164,165]. Among immunosuppressants, calcineurin-inhibitor based regimens were found closely linked with the development of these morbidities. Cyclosporine use was a risk factor for dyslipidemia and hypertension, whereas tacrolimus use was linked to post-LT diabetes by impairing insulin secretion, as discussed earlier^[166,167].

In an examination of all post-operative morbidity, Dare *et al*^[157] found similar rates of modified Clavien–Dindo grade 1 and 2 complications between NASH and non-NASH transplant recipients; however, NASH transplant recipients had increased rates of wound infections, bacteremia and pneumonia. Donor factors such as demographics, donation type (*e.g.*, donation after brain death or donation after circulatory death), BMI, cause of death, blood loss and transfusion requirement have not been found to be related to morbidity and mortality after transplantation amongst NASH recipients^[168,169]. Reported early reoperation rates for bleeding or biliary complications are around 15%, and re-transplantation rates are under 10%^[169,170].

NASH recurrence and post-transplant *de novo* NASH

The development of histologic NAFLD after LT has been well documented^[171]. Metabolic syndrome after LT predisposes recipients to recurrent and/or *de novo* NAFLD and NASH^[172]. The use of corticosteroids has also been implicated in the recurrence of NAFLD post-LT^[173]. At 2 years post-LT, around 60%-80% of recipients develop NAFLD, with at least grade 2 steatosis or above (34%-66% by biopsy). More extensive liver disease, such as NASH with progressive fibrosis (METAVIR stage ≥ 2 , defined as “more than septal formation”, including bridging fibrosis and cirrhosis), is rare however, occurring in only around 5% of recipients at 5 years post-LT^[162,171]. In a review of LT in 227 patients with NASH-related or cryptogenic cirrhosis, the probability of developing histologic hepatic steatosis after LT was 8.2%, 24.9% and

32.9% at 1, 5 and 10 years, respectively, but with only 6% developing recurrent NASH during the study period^[174].

Few studies have shown evidence of fibrosis beyond simple steatosis and early-stage NAFLD developing in the recipient allograft post-LT for NASH. In a recent single-center study, 88.2% of the 34 NASH recipients who had a liver biopsy post-LT had recurrent NAFLD, with 41.2% having evidence of recurrent NASH (median time from transplant of 47 mo)^[155]. Subgroup analysis demonstrated that patients with NAFLD/NASH had a significantly higher rate of impaired fasting glucose and hypertriglyceridemia than did recipients without recurrent NAFLD. In the same study, 87.5% of 56 recipients being evaluated with transient elastography had NAFLD (median time 75 mo). In this cohort, 81% of those with NAFLD recurrence had diabetes, compared with 51% of those without recurrence.

Histologic NASH has been shown to develop in the transplanted livers of NASH patients and has been documented as early as 6 mo post-LT^[175]. A major risk factor for the development of NASH is metabolic syndrome; one large series found NASH in 34% of recipient livers in patients who had metabolic syndrome compared with 13% in recipient livers of patients who did not exhibit metabolic syndrome^[120]. In the same study, hypertension and diabetes requiring insulin use were found to be significant risk factors for NASH recurrence—32% of hypertensive LT recipients developed NASH recurrence as opposed to 12% of those without hypertension; 37% of insulin users developed recurrence compared to 6% of non-users^[120]. Notably, the mean time from transplantation to documented NASH recurrence in this study was 18.2 mo^[120]. A separate study showed of NASH recipients showed 11% of allografts had progression from steatosis to steatohepatitis on serial biopsies post-LT with cumulative steroid exposure as a significant contributing factor to this progression^[173].

More recently, the unique entities of *de novo* NAFLD and NASH developing in transplanted livers have been recognized. A retrospective series of 68 LT recipients (84% transplanted for hepatitis C) reported development of *de novo* NAFLD in 18%, and 9% developed *de novo* NASH after transplantation^[176]. Development of *de novo* NAFLD/NASH could not be attributed to steatosis in the donor liver. Interestingly, a 10% increase in recipient BMI after LT correlated with a 35% increase in *de novo* NAFLD on biopsy. This study also found no significant effect of immunosuppressive regimens on the development of NAFLD. Conversely, a single-center retrospective review of 170 patients found that higher steroid dosage after LT contributed to the development of *de novo* metabolic syndrome in 33% of the study population, 50% of whom had *de novo* NAFLD within 1 year^[177].

The most common risk factors for post-LT *de novo* NASH are metabolic syndrome, PNPLA3 genotype, alcoholic cirrhosis, and the use of immunosuppressive agents, including tacrolimus and steroids^[153,162,178-180]. *De novo* NASH is most commonly recognized around 6 mo post-LT^[178,179]. Furthermore, the incidence of *de novo* NASH has been shown to increase from 30% at 1 year to 47% at 10 years^[180]. Importantly, no survival differences were found in patients with *de novo* NASH after LT who had more advanced fibrosis (F3 or F4 on transient elastography) compared to those with minimal or no fibrosis^[153,178,180].

There are little data on re-transplantation for recurrent or *de novo* NASH after LT. One single-center study reported 30% ($n = 6$) of recipients with NASH recurrence underwent re-transplantation—three patients had graft failure from recurrence, two had hepatic artery thrombosis and one had concomitant autoimmune hepatitis^[120].

Management recommendations

Management guidelines for post-LT patients with NASH are the same as those for non-transplant NASH patients, with emphasis on diet and exercise. Considering the propensity for NASH patients to develop metabolic syndrome after LT, careful attention should be placed on weight loss, strict glucose control and exercise^[181]. Management of obesity and hyperglycemia is crucial in the pre-transplant phase, as postoperative weight gain and metabolic complications are exacerbated by debility and immunosuppression^[152]. Considering the prevalence and mortality risk from cardiovascular complications post-LT for NASH, patients with cardiac comorbidities and risk factors should be diligently screened and managed in the pre-transplant phase^[157].

CONCLUSION

NAFLD/NASH cirrhosis is an increasingly frequent indication for liver

transplantation. The association of NAFLD/NASH with metabolic syndrome, cardiovascular disease and chronic kidney disease complicate the pre-and post-LT course and management. Physicians should appreciate the need for early optimization of transplant candidates to improve both pre- and post-transplantation survival. Multi-disciplinary teams which include dietitians, bariatric surgeons, endocrinologist, and other specialists could be important in the management of the unique problems facing this patient population.

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Pancreatic neuroendocrine tumors: Therapeutic challenges and research limitations

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Abstract

Pancreatic neuroendocrine tumors (PNETs) are known to be the second most common epithelial malignancy of the pancreas. PNETs can be listed among the slowest growing as well as the fastest growing human cancers. The prevalence of PNETs is deceptively low; however, its incidence has significantly increased over the past decades. According to the American Cancer Society's estimate, about 4032 (> 7% of all pancreatic malignancies) individuals will be diagnosed with PNETs in 2020. PNETs often cause severe morbidity due to excessive secretion of hormones (such as serotonin) and/or overall tumor mass. Patients can live for many years (except for those patients with poorly differentiated G3 neuroendocrine tumors); thus, the prevalence of the tumors that is the number of patients actually dealing with the disease at any given time is fairly high because the survival is much longer than pancreatic ductal adenocarcinoma. Due to significant heterogeneity, the management of PNETs is very complex and remains an unmet clinical challenge. In terms of research studies, modest improvements have been made over the past decades in the identification of potential oncogenic drivers in order to enhance the quality of life and increase survival for this growing population of patients. Unfortunately, the majority of systematic therapies approved for the management of advanced stage PNETs lack objective response or at most result in modest benefits in survival. In this review, we aim to discuss the broad challenges associated with the management and the study of PNETs.

Key words: Pancreatic neuroendocrine tumors; Gastroenteropancreatic neuroendocrine tumors; Management; Limitation; Novel Agents; Emerging targets

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Core tip: Pancreatic Neuroendocrine Tumors (PNETs) can cause severe morbidity due to excessive hormones production and overall tumor mass. The majority of approved therapeutic options in PNETs lack objective response suggesting that there is still a void in the understanding of the biology of this neoplasia. With the rising incidence and the underestimated prevalence of PNETs in the United States, it is paramount to discuss the challenges associated with the study and the management of this intractable disease for better patient outcomes. In this paper we elaborate on the comprehensive challenges and discuss novel and emerging therapeutic target in PNETs.

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INTRODUCTION

Physiologically, neuroendocrine cells receive neurotransmitter signals from the nervous system to secrete hormones in the blood to control many body functions^[1]. These specialized cells can be found in almost every organ of the body including the thymus, kidneys, prostate, skin, cervix, ovaries, testicles, stomach, colon, esophagus, appendix, small intestine, rectum, gallbladder, liver, and the pancreas^[2]. The pancreas is an essential organ involved in the digestive system and the endocrine system^[3]. In the endocrine system, pancreatic islet cells release hormones and polypeptides (including insulin, glucagon, somatostatin, amylin, pancreatic peptide, gastrin, incretin, and secretin) needed to regulate blood sugar level and multiple other body functions^[4]. When these hormonal producing cells of the pancreas become cancerous, they are termed Pancreatic Neuroendocrine tumors (PNETs)^[5].

Due to the advances in diagnostic modalities and the increase in awareness by oncologists and the general population, the incidence of PNETs is significantly increasing. According to the National Cancer Institute registry, the incidence of PNETs is estimated at 1000 new cases every year in the United States (<https://www.cancer.gov/types/pancreatic/hp/pnet-treatment-pdq>). However, the American Cancer Society has predicted that about 4,032 people will be diagnosed with PNETs in 2020 (<https://www.cancer.org/cancer/pancreatic-neuroendocrine-tumor/about/key-statistics.html>). In the past decades, PNETs have often been diagnosed at a later stage when the disease is already advanced or metastatic^[6]. In recent years, gastrointestinal oncologists are increasingly seeing patients diagnosed accidentally at an early stage^[7,8]. In this scenario, the tumor is further diagnosed using gallium 68 DOTATATE PET imaging coupled with a diagnostic quality contrast-enhanced MRI of the upper abdomen^[9]. The prevalence of the PNETs, that is the number of patients actually dealing with the disease at any given time is fairly high because the survival is much longer than pancreatic ductal adenocarcinoma (PDAC). In a retrospective study on about 1074 histopathological pancreatic specimens, Partelli *et al*^[10] examined whether the real prevalence of PNETs was underestimated. After excluding 284 patients who were diagnosed with PNETs as the main lesion, they found an incidental associated diagnosis of PNETs in 4% of the remaining specimens and they concluded that the frequency of incidental histological diagnosis of PNETs is considerably high and its prevalence is probably underestimated^[10].

In general, tumors grade and classification are the fundamental basis for neuroendocrine tumors (NETs) therapeutic decisions^[11]. Tumor grade is a system used to predict how fast tumors would grow/spread and differentiation is a key feature to predict their behavior^[12]. Ki-67 (MIB1) only stains actively dividing cells and not resting cells, is most commonly used to establish the grade of the tumor; thus, more dividing cells implies more aggressive PNETs. The world health organization classifies PNETs into three main categories based on the Ki67 proliferation index and/or mitotic count per 10 high power fields. Well-differentiated PNETs (also known as panNETs) are classified as Grade 1 (low grade), Grade 2 (intermediate grade), and Grade 3 (high grade) with a Ki67 index of < 2%, 2%-20%, and > 20% respectively. Poorly differentiated PNETs (also referred to as panNEC) are categorized as grade 3 (high grade) with a Ki67 index greater than 20%^[13]. Also, tumor grade strongly predicts

outcomes such as how fast the tumor will grow and how long it can be controlled with therapy. For well-differentiated grade 1, meaning PNETs patients who have small low-grade tumors, oncologists often wait and do not operate (watchful waiting protocol) and most recently treat with the PRRT (Peptide receptor radionuclide therapy), a treatment that is well tolerated and very safe (the latter drug will be further discussed in this manuscript)^[14]. Well-differentiated grade 3 PNETs are fairly indolent but often have an unpredictable course and behave similarly to grade 2 panNETs; poorly differentiated grade 3 panNEC are aggressive^[15]. It is important to note that there exists an additional category for PNET termed: Mixed neuroendocrine-non-neuroendocrine neoplasm (MiNEN)^[16,17].

Recently, cross-species analysis of mice and human panNET tissues illustrate the existence of three molecular subtypes of PNETs including Islet/Insulinoma tumors [IT (less aggressive, and express genes associated with differentiated mature β -cells)]; metastasis-like/primary [MLP (invasive and express genes associated with immature β -cells, and stem cells)], and intermediate (express genes similar to IT and are moderately invasive)^[18]. Next-Gen sequencing illustrates that commonly mutated genes associated with neoplasia pathogenesis are not significantly implicated in PNET development and progression^[19]. However, hyperactivation of PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways have been well documented to be the main regulators of proliferation in NETs^[20]. Frequent mutations in multiple endocrine neoplasia 1 (MEN1; 44%), death domain-associated protein (DAXX)/chromatin remodeler (ATRX; 43%), mTOR (15%) pathway genes, and Von Hippel Lindau (VHL) alongside several other hereditary disorders are observed in PNETs^[21]. Loss of function of the tumor suppressor gene PTEN is frequently found in PanNETs and is responsible for the over-activation of the PI3K-Akt-mTOR cascade^[22]. A new examination using whole-genome sequencing of 102 primary PNETs illustrates that germline mutations in DNA repair genes such as MUTYH, CHECK2, and BRCA2 were noted in sporadic PNETs^[23].

Compared to other gastroenteropancreatic neuroendocrine tumors (GEP-NETs), PNETs are a very heterogeneous subtype of cancers with unique pathophysiological features that constitutes a major challenge in the management of this neoplasia^[24]. Multiple factors impede the management and the study of PNETs. As mentioned above, the majority of patients are diagnosed at a later stage when the disease is already advanced due to the lack of specific biomarkers and disease-associated symptoms^[6]. Systemic treatments for PNETs only stabilize the disease most likely because of inherent and acquired drug resistance. Another challenge in drug development is related to the poor delivery of therapeutic agents related to the location of the pancreas^[25]. Lack of reliable preclinical models (mostly cell lines) limits the ability to rapidly test promising therapies. The small population of relevant candidates with PNETs is a major challenge for conducting larger clinical trials^[26]. Immunotherapy is not an option for this patient population given that the pancreas appears to be an immunologically coldsite^[27,28]. Despite significant increase in the incidence of PNETs in the United States, this disease remains an understudied and underfunded area of research. This review intends to discuss the major challenges associated with the management of PNETs in the clinic and highlight research limitations associated with its study.

CURRENT THERAPEUTIC OPTIONS

The ultimate question in the management of PNETs is when to give specific treatment to a patient; keeping in mind that one size cannot fit all. This is left to the clinician's own assessment as to who should have surgery? Who are the ideal candidates for drug X? And who are the ideal candidates for chemotherapy? Systemic therapeutic decisions for the management of PNETs must be personalized and rely on various considerations including functional imaging and molecular profiling in addition to clinical considerations such as hormonal secretion, tumor grade, disease burden, and the rate at which tumor progresses^[29]. These considerations predict whether systemic or locoregional treatment will benefit patients^[30]. The impact of therapies on the quality of the life of the patient must be considered prior to making any therapeutic decision. More than 80-90% of pancreatic islet tumors express somatostatin receptors (SSTRs). These SSTRs are G protein-coupled seven transmembrane receptors that control cellular proliferation and hormone production by PNET cells and as such are targets for diagnostics and therapeutics (theranostics)^[31-33]. The somatostatin analogs (SSRA) Octreotide and Lanreotide (targeting specifically SSTR2 and SSTR5) are

commonly used for initial treatment of advanced stage well-differentiated grade 1 or 2 PNETs^[34]. Somatostatin analogs can inhibit hormone production from PNETs. For example Octreotide and Lanreotide can be used to prevent hypoglycemia in patients with positive SSIR2; however, these drugs could worsen hypoglycemia in patients not expressing SSIR2^[35]. In addition, SSRA are also used as palliative treatments to slow down the progression and stabilize the disease burden^[36]. However, somatostatin analogs do not cause tumor shrinkage. Peptide Receptor Radionuclide Therapy (PRRT), everolimus (mTOR inhibitor), chemotherapy or sunitinib (multi RTK inhibitor) are used to manage well-differentiated PNETs that have progressed on SSRA^[37,38]. A combination of chemotherapeutic agents (such as Capecitabine + Temozolomide or platinum-based regimens) constitutes the first-line treatment for panNEC, MiNEN, and metastatic disease^[39]. PRRT is also relevant for metastatic disease. Unfortunately, most of these treatment strategies used by GI oncologists to overcome tumor burden lack objective response and PNETs remain a serious unmet problem in the clinic. At most, these therapies stabilize the tumors and do not enhance the overall survival of patients (Figure 1).

THERAPEUTIC OPTIONS IN PNETS ONLY STABILIZE THE DISEASE

The management of NETs and PNETs, in particular, is greatly personalized and requires expert multidisciplinary strategies including surgery, medical oncology, endocrinology, radiation oncology, cardiology, gastroenterology, pathology, interventional radiology, diagnostic radiology, and nuclear medicine^[40]. The majority of FDA approved drugs for the management of PNETs lack objective response characterized by meager progression free survival (PFS) and inability to shrink tumors in the clinic^[41]. Yao *et al*^[42] showed that the median progression free survival with everolimus as a single agent treatment for PNET patients is estimated at 11.0 mo relative to 4.6 mo with placebo. The overall survival (OS) with everolimus was estimated at 44.0 mo relative to 37.7 mo with placebo^[43]. This means everolimus stabilizes PNETs progression for an average of 6.4 mo. Similarly, Faivre *et al*^[44] and Vinik *et al*^[45] showed that the PFS with sunitinib is evaluated at 11.4 mo relative to 5.5 mo with placebo. The CLARINET study designed to evaluate the response of Lanreotide in metastatic enteropancreatic neuroendocrine tumors showed that this drug can only stabilize the progression of neuroendocrine tumors^[46-49]. In the same manner, the PROMID study shows that Octreotide can only lengthen the time to tumor progression in functional and metastatic NET patients^[50]. Exner *et al* recently showed that Octreotide does not inhibit the growth of multiple NET cell lines including BON-1 and QGP-1 the commonly available PNET cellular models^[51]. The CAPTEM study (Capecitabine in combination with Temozolomide) shows that PNET patients achieved a median PFS of 13 mo^[52]. Dilz and colleagues analyzed data from 96 advanced PNET patients treated with Streptozocin + 5 FU; they found that 40.6% of patients showed stable disease while 16.7% showed progressive disease^[53]. Nevertheless, in terms of treatment strategy, the PRRT, appear to be the most promising treatment for PNETs. PRRT is a drug coupled with therapeutic radionuclide lithium 177 and is injected intravenously irradiates PNET cells directly and radiates^[54]. PRRT treatment results in PNET shrinkage; nevertheless, this treatment also stabilizes the tumor for a long period of time^[55]. The side effects of PRRT are very mild, there can be some nausea with the treatment at the time of the administration which is much related to the IV fluid that is given to protect the patient's kidneys^[56].

HETEROGENEITY IN PNETS

PNETs' heterogeneity is considered the major challenge in the management of this specific type of neoplasia in the clinic^[57,58]. As mentioned above, pancreatic islet cells are specialized entities that participate in the endocrine function of the pancreas by releasing hormones and peptides necessary to maintain body homeostasis. PNETs can be functional or non-functional depending on whether they release these hormones^[59,60]. Functional PNETs release excess hormones leading to a variety of hormonal associated symptoms. For example, Insulinoma can release excessive insulin, which results in hypoglycemia and related symptoms^[61]. Insulinomas are mostly benign, < 10% are malignant; this subtype of PNETs can mostly be removed by surgery, but liver metastasis patients have < 2% survival^[62]. In Gastrinoma (representing 30% of all PNET), excessive gastrin release would cause Zollinger-

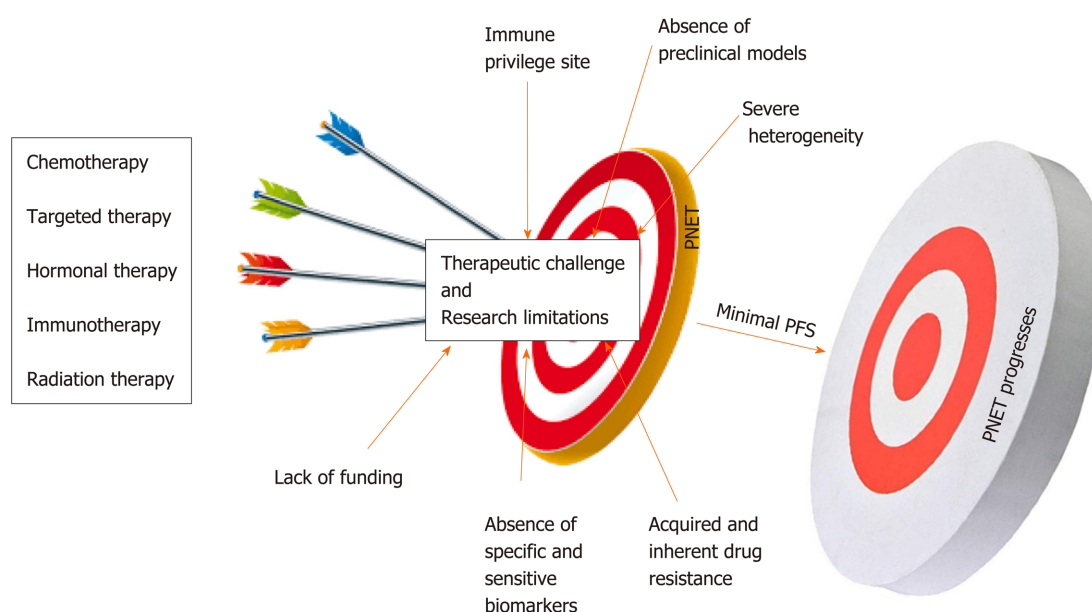


Figure 1 Graphical abstract. PFS: Progression free survival; PNET: Pancreatic neuroendocrine tumor.

Ellison syndrome characterized by increased acidity of the stomach and could eventually result in severe peptic ulcer disease and chronic diarrhea (surgery is the only potential cure for tumor > 2 cm followed with PPI)^[63]. It is important to note that gastrinoma can also be found in the duodenum but there are much smaller than those from the pancreas. The last example will be VIPoma, in which vasoactive intestinal peptide is aberrantly released causing severe diarrhea and associated symptoms; however, this specific type of functional PNET is very unusual^[64]. Thus PNET release hormones, which make the patients sick not the tumor itself. Meanwhile, non-functioning PNETs do not develop hormonal symptoms because they produce insignificant amount of hormones that lack clinical implication. The majority of PNET is nonfunctional and is often diagnosed when the disease is unresectable, advanced or metastatic^[6]. Attempts to manage tumor burden and hormonal symptoms concomitantly may constitute a challenge in the clinic. PNETs can also form a large mass, which causes pain by pushing on nerves at the pancreas. Additionally, patients could have a small tumor that has spread to the liver and the liver starts to fill up with metastasis and that may cause severe pain. Liver metastasis is the most significant prognosis factor in PNETs progression setting^[65]. The poor quality of life due to the severity of pain in these patients could also alter the effectiveness and the treatment outcomes. As described above a wide range of malignant phenotypes characterizes PNETs' clinical heterogeneity. Malignant phenotypes in PNETs range from slow-growing (almost indolent), noninvasive tumors, locally invasive and metastatic tumors. Slow-growing tumors are often observed (watchful and waiting) and do not require any therapeutic intervention; however, PNETs have the potential to acquire aggressive phenotype when they reach a certain size and monitoring these tumors for prompt intervention is not an easy task^[66].

PNETs can be sporadic or associated with a genetic syndrome. Genetic syndromes associated with PNETs include Multiple Endocrine Neoplasia type 1 (MEN 1), Von Hippel Lindau (VHL), Neurofibromatosis (NF), and Tuberous Sclerosis Complex (TSC)^[67,68]. Syndrome associated PNETs demonstrate a significant challenge in the clinic when considering how to best manage patients. For instance, MEN1 functional PNETs patients can undergo tumor resection with a high cure potential; meanwhile, surgery is not a therapeutic option for MEN1 nonfunctional PNETs^[69]. As mentioned above, nonfunctional PNETs are often diagnosed at a later stage, with multiple sites within the pancreas (when they are not metastatic); they are small tumors, thus, require resection. Moreover, MEN1 nonfunctional PNETs are often associated with diabetes; therefore, pancreatic resection is not advised especially in young patients^[70].

PNET LACK SPECIFIC AND SENSITIVE BIOMARKERS

A biomarker is a measurable biological indicator of the presence or severity of diseases. In cancer management biomarkers have critical importance; they are necessary for prognostication and highly essential to ease early diagnosis^[71]. More importantly, biomarkers are necessary to predict and monitor response to specific treatment including recurrence after surgical intervention^[72,73]. Lack of adequate biomarkers is another fundamental problem in the management of this disease in the clinic. Current PNET serum based biomarkers such as chromogranin A (CgA), pancreatic peptide (PP), and neuron specific enolase (NSE) have limited sensitivity and specificity^[74,75]. The sensitivity and the specificity of a good biomarker should be greater or equal to 90%. The sensitivity of CgA range from 60 to 83% and its specificity ranged from 72 to 85%^[76,78]. Meanwhile, the sensitivity of PP range from 31 to 63% and its specificity is approximately 67%^[79,80]. The sensitivity of NSE is 33% and its specificity is 73%^[81,82]. This poor sensitivity and specificity could be an explanation for the use of PNETs grade and stage as prognostic biomarkers for this neoplasia^[83]. Additionally, CgA is an unspecific biomarker given that it can be released by non-neuroendocrine tumors including gastric disorder, inflammatory bowel disease, end-stage renal disease (ESRD), and obstruction of blood vessels (cardiovascular disease)^[84,85]. NSE is also a non-specific biomarker because increase level of this molecule is associated with brain injury^[86]. Nevertheless, vasostatin-1 (VS-1): The N-terminal fragment of chromogranin A (CgA), has recently been identified to be more accurate than CgA as neuroendocrine biomarker and the plasma levels of VS-1 are not altered by proton pump inhibitors (PPI) used in gastrinoma^[87].

PNETS LACK SIZABLE NUMBER OF PRE-CLINICAL CELLULAR MODELS

The development of novel anticancer drugs necessitates the development and use of appropriate and relevant representative *in vitro* and *in vivo* models. Lack of reliable PNETs' cell lines holds back meaningful research and has significantly disadvantaged the management of PNETs for decades^[88,89]. Significant strides have been made over the past four decades to develop cellular and mouse models of PNETs. Currently, there are only a few PNET cellular models available for biomedical research^[90,91]. BON-1, QGP-1, and CM are the available PNET cell lines often used in research to study this disease. Twenty-five years ago, Townsend *et al*^[92] established BON-1 cell line from the lymph node of a 28-year-old male. QGP-1 is a functioning PNET cell line established in the 1980s from a 61-year-old male^[93]. BON-1 and QGP-1 cells were recently authenticated to belong to neuroendocrine and epithelial lineage, but their molecular characterizations do not often resemble those seen in patients' primary cancers. For instance, exome sequencing and genome-wide copy number analysis reveal that BON-1 and QGP-1 do not harbor PNET-associated mutations such as mTOR, DAXX/ATRX, MEN1, VHL, and NF; questioning the relevance of using these models for PNET study^[94,95]. The fast growing potential of these two cell lines does not reflect the slow growth phenotype of most PNETs^[96]. In general neuroendocrine cancers are characterized by high expression levels of somatostatin receptors; however, BON-1 and QGP-1 define a very low expression of somatostatin receptors^[51]. Kim, B.L. and colleagues have recently shown that BON-1 and QGP-1 illustrate similar characteristics of immature/non-functional pancreatic β/δ -cells or pancreatic endocrine progenitors. They show that BON-1 and QGP-1 display high expression levels of NEUROG3 and FOXA2 two genes associated with immature/non-functional pancreatic β/δ -cells and pancreatic endocrine progenitor, respectively^[97]. The latter suggests that these two cell lines have acquired malignant transformation at an early stage of their development. The latter also suggests that QGP-1 may not be functioning (gastrinoma) PNETs as previously characterized. Bente *et al*^[98] established and characterized a novel lymph node-derived cell line (NT-3) from a male patient with well-differentiated PNETs. NT-3 cells are specifically insulinoma (the most common functional PNETs) and express neuroendocrine characteristics that surpass the phenotype observed in BON-1 and QGP-1. Even though NT-3 could become a relevant model for functioning PNETs, this cell line has not yet made any meaningful impact in the study of this intractable disease and only two studies has been published using these cells hitherto.

Several mouse models of PNETs have been developed throughout the years. It has been well established that MEN1 syndrome is associated with the development of PNETs. Therefore conventional MEN1 loss mouse model has been developed to

successfully characterized PNETs. For instance, Bertolino and his team have demonstrated that heterozygous *MEN1* mutant mice develop a range of endocrine tumors often seen in multiple endocrine neoplasia type 1 patients^[99]. Moreover, Shen and colleagues have developed the *MEN1*-PDXCre mouse model to illustrate that loss of the expression of menin via knockout of *MEN-1* in mature pancreatic endocrine cells resulted in tumor development^[100]. Here, they confirm an association between *MEN1* syndrome and the development of PNET lesions. Likewise, Li *et al*^[101] have developed *Men1*^{fl/fl}-RipCre⁺ mouse model in which *MEN1* ablation in pancreatic β -cell decreased the expression of critical transcription factor and resulted in the development of glucagonoma one of the rarest PNET subtypes. The latter mouse models have been significantly important to successfully characterize PNETs. The RIP1-TAG2 mouse in which PNETs are induced by expression of SV40 T-antigen in the beta cells of Langerhans has been used as a relevant mouse model for PNETs^[18]. However, there is a significant concern with RIP1-TAG2 mice because the viral system used to induce PNETs abrogates *TP53* and *RB* genes that are rarely seen in PNETs. To complement the RIP1-TAG2 mouse model, Chung Wong *et al*^[102], proposed [GEMMs-MPR (*Men1*^{flox/flox} Pten^{flox/flox} RIP-Cre)] and MPM (*Men1*^{flox/flox} Pten^{flox/flox} MIP-Cre) as novel mouse models for PNETs. At this point it is too early to assert the relevance of these two models for PNETs therapeutic examination. Here, we argue that there is a need to invest more in developing PNETs cellular models in order to enhance our understanding of the progression of PNETs. The study and analysis of patients' tissue by researchers are fundamental for cancer research in general. Research on patient tissue could offer critical information necessary to prevent, diagnose, and more importantly treat cancer patients. However, lack of access to patient tissues also constitutes a barrier to study PNETs.

EMERGING NEW THERAPEUTIC TARGETS IN PNETS

Cysteine-rich angiogenic inducer 61

The CCN1-6 is a family of six extracellular associated proteins known to play a critical role in cellular processes including cell adhesion, migration, proliferation, differentiation, survival, apoptosis, and senescence^[103]. This family of matricellular proteins contains: Cysteine-rich angiogenic inducer 61 (CYR61) or CCN1, CTGF or CCN2, NOV or CCN3, WISP1 or CCN4, WISP2 or CCN5, and WISP6 or CCN6^[102]. Upon secretion in the extracellular matrix, CYR61 binds directly to various integrin receptors in a cell type-dependent manner^[104]. It is important to note that human's and mouse CYR61 protein share a 98.2% sequence identity^[105]. Several studies have suggested the implication of CYR61 in tumorigenicity and progression. For instance, Huang *et al* have shown that CYR61 promotes breast cancer lung metastasis through tumor cell extravasation and suppression of anoikis^[106]. The authors argued that CYR61 support lung metastasis by regulating two critical events relevant to the late steps of metastatic dissemination including enhancement of extravasation of cancer cells into the lung and, secondly, inhibition of process of anoikis via the activation of AMPK α pathway but not through AKT, FAK or ERK1/2 signaling. Recently, Habel and colleagues have illustrated that CYR61 induces metastatic spreading through IGF1R β -dependent EMT-like process in osteosarcoma^[107]. It is well known that a large number of PNETs are metastatic at presentation (40-80%) and liver metastasis (about 40-90%) is the most significant prognosis factor in PNETs progression. Thus, targets associated with metastasis/invasion could be an attractive area to manage this disease. Also, relevant to pancreatic cancers, it has been shown that CYR61/CCN1 signaling facilitates pancreatic carcinogenesis via activation of mechanisms of EMT and stemness^[108]. In this study, Haque and colleagues illustrate that in PDAC, CYR61 transcripts and proteins increase as the disease progresses. More significantly, Maity *et al*^[109] have recently shown that CYR61 regulates dCK and CTGF causing Gemcitabine-resistance in PDAC. First, they show that CYR61 is highly activated in PDAC and correlates with Gemcitabine resistance. They also show that ablation of CYR61 sensitizes PDAC cellular models to Gemcitabine in 2D and 3D culture. The latter suggest that CYR61 is implicated in PDAC drug resistance, which is a major factor for therapeutic failure in PNETs. Thus, what is the implication of this target in the setting of PNETs development and progression? A novel study has suggested that CYR61 may be a tumor-promoting gene in PNETs. Notably, Huang and colleagues have newly shown that CYR61 interferes with normal pancreatic architecture and promotes PNETs progression^[110]. They crossed Rip1CYR mice with Rip1-TAG2; the resulting Rip1Tag2CRY mice developed β -tumors significantly larger, more invasive

and more vascularized compared to β -tumors in the Rip1-Tag2 mice (keeping in mind that CYR61 is highly conserved in human and mice). The latter study demonstrates that CYR61 is viable target in the complex to treat PNETs and required further clinical examination.

Forkhead box protein M1

Forkhead box protein M1 (FOXM1) is a critical proliferation-associated transcription factor found to be increasingly and spatiotemporally expressed during the highly regulated cell cycle events^[111]. Several studies have suggested that FOXM1 is closely involved with the processes of cell proliferation, self-renewal, and tumorigenesis^[112-114]. FOXM1 is differentially expressed in typical carcinoids relative to atypical carcinoids cells and more importantly, FOXM1 expression was significantly different in large cell neuroendocrine carcinomas compared to small cell lung cancers^[115,116]. In a recent study, Franziska *et al*^[117] have shown that FOXM1 expression is linked to proliferation, differentiation and metastasis in GEP-NETs and that inhibition of FOXM1 is a potential new therapeutic option for these intractable subtypes of cancers. Utilizing Genome-wide expression profiling on biopsies from well-differentiated neuroendocrine tumors of the distal ileum and metastatic disease at the time of diagnostic, Ellinor Andersson *et al*^[118] have shown that FOXM1 expression is upregulated in small intestinal neuroendocrine tumors. The latter studies illustrate that FOXM1 has a significant implication in the development and progression of NETs. This is also true for PNETs, as De Rycke *et al*^[119] have shown that FOXM1 expression defines highly proliferative group of tumors in pancreatic neuroendocrine tumors and pulmonary neuroendocrine tumors. They also showed that the Thiostrepton (FOXM1 specific inhibitor) display a strong anti-tumor effect in (BON-1, and QGP-1), and H-227, pancreatic and pulmonary neuroendocrine cell lines, respectively.

UPR coordinator proteins IRE α and PERK

It has been well established that increased protein translation, accumulation of unfolded/misfolded proteins, and several other dynamic changes in the cells microenvironment can activate endoplasmic reticulum (ER) stress and promotes the unfolded protein response (UPR) that aide cell survival^[120]. Nevertheless, sustained ER stress could lead to ER-associated programmed cell death. Inositol-requiring enzyme 1 α (IRE1 α) and protein kinase R-like endoplasmic reticulum kinase (PERK) are two of the major coordinators of the UPR response^[121]. Activation of the latter has been shown in several cancers and linked to oncogenesis, tumor growth, metastasis and chemoresistance. Croft A *et al*^[122] have illustrated that mutant BRAF (V600E) promotes IRE1 and ATF6 activation in melanoma cellular models. Additionally, Hart *et al*^[123] have shown that activation of c-MYC in mouse embryonic fibroblasts induces IRE1 and PERK activation. Moreover, Blazanin *et al*^[124] have recently demonstrated that RAS activation was followed with UPR activation in melanocytes and keratinocytes. All these studies suggest that UPR coordination proteins are very much likely to promote cancer progression. Another recent publication demonstrates that secretory factors from endoplasmic-stressed cells aided survival of nearby cells to cytotoxic agents via UPR activation^[125]. It is well known that islet cells in the pancreas secrete hormones and polypeptides that could sensitize these cells to elevated ER stress. Therefore, sustain ER stress coupled with hyper activation of UPR could be a major mechanism regulating PNET tumor growth and/or drug resistance. More importantly, a recent study has revealed that the expression level of key proteins such as BiP, CHOP, ATF4 involved ER stress are significantly upregulated in PNET and that this hyperactivation was associated with advanced clinicopathological features^[126]. The authors of the latter study used immunohistochemical analysis by tissue microarray of 49 human PNET tissues and found that BiP, CHOP, ATF4 were significantly upregulated compared to normal tissues. They also show that high expression of Bip was significantly associated with high grade tumor, proliferation and poor survival. Finally, Moore and colleagues published an excellent paper illustrating the implication of UPR signaling in PNETs growth and survival. Using available mouse models for PNETs including RIP1-TAG2 mouse model, they specifically show that UPR is upregulated in this disease and inhibition of UPR cascade significantly reduces tumor growth^[127].

Nicotinamide phosphoribosyltransferase

In general, cancer cells often develop strategies to promote their survival under stressful conditions caused by the administration of anticancer therapeutics. As mentioned above, PNETs are known to be equipped with intrinsic drug resistance

mechanisms that alter the efficacy of personalized or systemic therapies. The protein nicotinamide phosphoribosyltransferase (NAMPT), best known as the rate-limiting enzyme involved in the salvage pathway of Nicotine Adenine Dinucleotide (NAD) biosynthesis in mammals^[128] could become a novel target for therapy-resistant PNETs. NAD is a critical redox coenzyme that is essential for multiple physiological processes including DNA repair, oncogenic signal transduction, transcription, genomic integrity, and apoptosis^[129]. Three different pathways govern the biosynthesis of NAD in mammals. The essential amino acid tryptophan is the precursor of the *de novo* pathway that includes 9 steps in which the tryptophan is converted into quinolinic acid that is further metabolized into NAD⁺^[130]. This *de novo* pathway of NAD synthesis includes multiple steps and requires more energy; thus, most cancers cells rely on the alternative pathway of NAD synthesis. The alternative pathways of NAD biosynthesis are termed NAD salvage pathway and the Preiss-Handler pathway^[131]. Nicotinate phosphoribosyltransferase (NAPRT1) is the rate-limiting enzyme in the Preiss-Handler pathway. In this pathway, Niacin (also known as Nicotinic acid or Vitamin B3) is converted into Nicotinic acid mononucleotide (NMN) by the nicotinate phosphoribosyltransferase (NAPRT), and then NMN is converted into Nicotinic acid adenine dinucleotide (NAAD) that is finally converted into NAD by the enzyme NAD synthetase^[132]. NAPRT1 is often lost in cancer; thus, the salvage pathway, governed by NAMPT is preferably used in cancer; making NAMPT a potential therapeutic target for the management of cancers. In the salvage pathway, Nicotinamide (an additional form of vitamin B3) is converted into Nicotinamide mononucleotide by the rate-limiting enzyme NAMPT in the presence of the phosphoribosyl pyrophosphate (PRPP); next, the nuclear Nicotinamide Mononucleotide Adenylyltransferase (NMNAT) further converts the NMN into NAD^[131]. NAMPT biological function is not limited to the regulation of total cellular and mitochondrial levels of NAD necessary for cell survival. NAMPT also exhibits growth factor activity in this regard, it is called Pre-B cell colony enhancer factor (PBEF)^[133]. Evidence has also shown that NAMPT has a hormonal activity, it's called Visfatin (therefore named Visafatin)^[134]. NAMPT could also be an adipocytokine and called Insulin-mimetic hormone; however, this adipocytokine function is the object of controversy hitherto. When located in the cytoplasm (intracellular milieu) iNAMPT has an enzymatic function mainly the catalysis of the salvage pathway of NAD^[129]. Outside the cell (extracellular milieu or circulating in the plasma), eNAMPT presumably plays the role of growth factor, and hormone (PBEF, Visfatin respectively) and allegedly adipocytokine (Insulin-mimetic hormone)^[132,134]. NAMPT is a 52-kDa molecule with a length of 36.908 base pairs encoded by the NAMPT gene located at the 7q22^[129]. Human NAMPT's crystal structure alone or in complex with nicotinamide was determined at 2.1 Å resolution by the selenomethionyl SAD method^[135]. Tao Wang *et al*^[136] described the crystal structure of NAMPT as a dimeric type II phosphoribosyltransferase homolog of NAPRT1. NAMPT comprises 491 (including initial methionine) amino acids and its active site includes an Asp 219 that forms a hydrogen bond with Nicotinamide^[131]. Over the past three decades, multiple studies have illustrated the involvement of NAMPT in numerous malignancies^[137-138]. An important number of cancers including PNETs^[139] show increased expression of NAMPT; however, the mechanism associated with NAMPT upregulation is unknown. In a recent study, Alvarez M.J. and colleagues evaluated more than 200 patient cohort of GEP-NETs^[140]. They showed that NAMPT is one of the mechanistic dependencies of neuroendocrine tumors. This paper looked at the responsiveness of GEP-NET cell lines to different agents and found that NAMPT inhibition can impact their proliferation. The findings of this comprehensive study support the fact that NAMPT is critical for GEP-NET survival. Additionally, Michael Ohanna and colleagues have shown that NAMPT regulates drug resistance and invasive phenotype in melanoma^[141]. In the forthcoming passages we will discuss the utility of targeting NAMPT in PNET using small molecule drugs (Figure 2).

P21-activated kinase 4

P21-activated kinase 4 (PAK4) is a member of a family of serine-threonine kinases that play a role in both oncogenesis and cancer progression^[142,143]. PAK family members are key effectors of the Rho family of GTPases (a sub family of the Ras superfamily), which act as regulatory switches that control critical cellular processes such as motility, proliferation, and survival^[144]. The latter indicates that PAK4 is the downstream effector of Ras activity that promotes growth and proliferation in PNETs; thus making PAK4 a relevant target for this disease. P21-Activated kinase name arose following their identification as effectors of Rho GTPases (*e.g.*, CDC42 and Rac), each of which is 21 kDa in size. Upon activation by mutation or overexpression, the majority of Pak isoforms (Group I PAK 1,2,3 or Group II PAK 4,5,6) have oncogenic signaling effects.

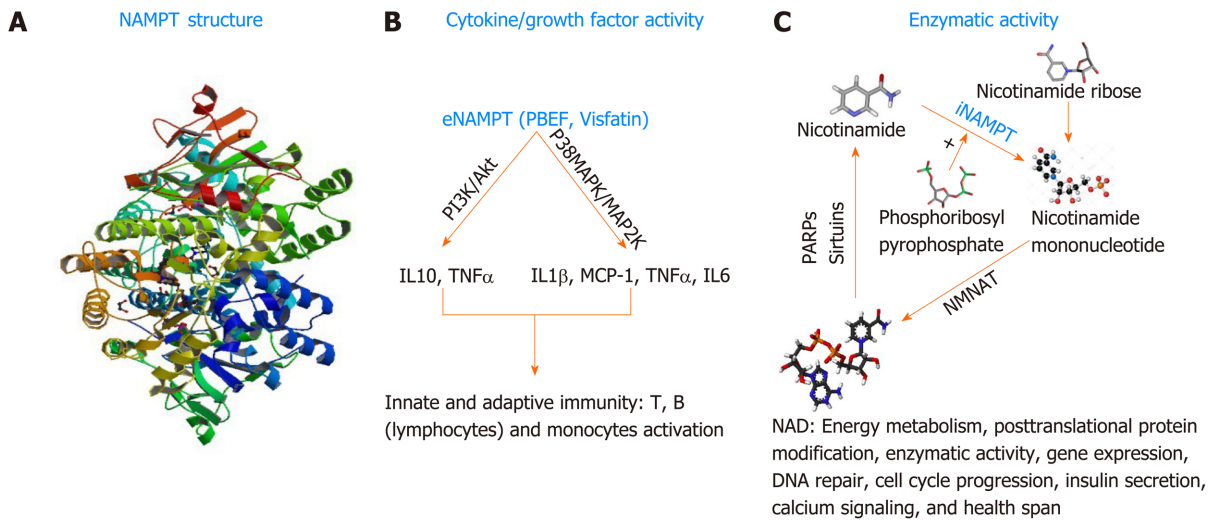


Figure 2 Nicotinamide phosphoribosyltransferase's biological functions. A: Structure of nicotinamide phosphoribosyltransferase (NAMPT). Structure obtained from RSCB Protein Data Bank, Deposited: 2008-06-15 Released: 2009-08-18. Deposition Author(s): Ho, M., Burgos, E.S., Almo, S.C., Schramm, V.L.; B: NAMPT and immune signaling; C: NAMPT signaling in NAD biosynthesis. NAD: Nicotinamide adenine dinucleotide; NAMPT: Nicotinamide phosphoribosyltransferase; eNAMPT: Extracellular Nicotinamide phosphoribosyltransferase; iNAMPT: Intracellular nicotinamide phosphoribosyltransferase; NMNAT: Nicotinamide mononucleotide adenyltransferase; TNF α : Tumor necrosis factor alpha; IL: Interleukin; MCP-1: Monocyte chemoattractant protein-1; PARP: Poly(ADP-ribose) polymerase.

As previously mentioned, PAK4 is a key effector of Cdc42 (cell division control protein 42 homolog) and Rac1 (Ras-related C3 botulinum toxin substrate 1); thus, acts as a critical mediator of the RhoA family of GTPases^[145]. Pertinent to pancreatic neoplasia, earlier studies have shown that copy number alteration analyses illustrate increased expression of PAK4 in pancreatic ductal adenocarcinoma (PDAC) patients^[146]. Hyperactivity of PAK4 has been implicated in cancer progression by activating oncogenic signaling pathways, such as RAF/MEK/ERK and PI3K/AKT^[147-149]. Additionally, other investigations have also linked PAK4 overexpression to cell migration, cell adhesion, and anchorage-independent growth^[150]. PAK amplification can cause the activation of markers associated with drug resistance in PNETs including Akt, ERK, mTORC1, mTORC2^[151], β -catenin, and IGF-1^[152]. PAKs have also been shown to promote FAK (additional drug resistant molecule in PNETs) by this means it enhances cell migration and metastasis in breast carcinoma models^[153]. Our group has demonstrated that PAK4 knockdown by means of siRNA inhibits the growth of PNETs cellular models (QGP-1 and BON-1)^[139].

NAMPT and PAK4 inhibition

For decades, PAK4 and NAMPT have remained non-drugable targets. The adenosine-triphosphate (ATP) binding cleft in PAK4 is a flexible hinge structure, which does not allow the development of effective inhibitors^[154]. The first PAK4 small molecule inhibitor PF3578309 (IC₅₀: 1.3nm in cell-free assay) is an ATP competitive Type I and pyrrolopyrazole inhibitor of PAK4 failed to move in advanced clinical trials for cancer management. PF3578309 failed clinical study because it happened to be a PGP substrate. Among all NAMPT inhibitors, only two: APO866/FK866, and GMX1777 (GMX1778/CHS828), were evaluated in phase I clinical trials. Unfortunately, further evaluations were discontinued predominantly due to undesired dose-limiting toxicities. APO866 is the first developed NAMPT inhibitor with an IC₅₀ varying between (0.09 and 27.2 nm in cell-free based assay^[155]). It had been well established that APO866 inhibits proliferation and growth in a wide variety of human cancers *in vitro* and *in vivo*. For instance, in 2003 Hasmann *et al*^[156] showed that inhibition of NAMPT using APO866 is a novel mechanism to induce apoptosis in leukemia. At exactly the same time, Dreves *et al*^[157] were the first to illustrate the antiangiogenic properties of APO866. These two pilot studies lead to a phase I/II trial (NCT00435084) opened in the United Kingdom to investigate the safety and tolerability of APO866 for the treatment of refractory chronic lymphocytic leukemia (<https://clinicaltrials.gov/ct2/archive/NCT00435084>). Phase II study (NCT00432107; and NCT00431912) of APO866 were opened at four locations (Austria, France, Germany, and Switzerland) to define its efficacy and safety for the treatment of melanoma and cutaneous T cell

lymphoma <https://clinicaltrials.gov/ct2/show/NCT00432107>, and <https://clinicaltrials.gov/ct2/show/NCT00431912>, respectively. The primary outcome measure of APO866 in these studies lacked objective responses (pharmacodynamics) and the dose limit toxicity was found to be thrombocytopenia^[158]. GMX1777 (EB1627) is a water-soluble prodrug of the GMX1778 a cyanoguanidine compound that selectively inhibits NAMPT with an IC₅₀ of less than 100 nm in cell-free assay^[159]. Two trials conducted by Gemin X pharmaceutical had investigated this drug for anticancer therapy. Firstly, GMX1777 was evaluated for safety and efficacy in phase I clinical trial (NCT00457574) for the treatment of refractory solid tumors and lymphomas <https://clinicaltrials.gov/ct2/show/NCT00457574>. Secondly, GMX1777 was evaluated in phase I/II study in combination with Temozolomide (an oral chemotherapy drug) for the treatment of metastatic melanoma <https://clinicaltrials.gov/ct2/show/NCT00724841>.

KPT-9274: Available PAK4-NAMPT dual inhibitor

Recently, Karyopharm Therapeutics Inc. developed KPT-9274 a first in class orally bioavailable small molecule inhibitor which targets PAK4 and NAMPT^[139]. KPT-9274 is a distinct class of allosteric modulator that binds to the kinase domain of PAK4. Most importantly, the latter investigational drug is not a PGP substrate. It is important to know that the drug KPT-9274 has been established to be a dual inhibitor of PAK4 and NAMPT^[160,161]. Senapedis *et al* used stable isotope labeling of amino acids in cells (SILAC) to illustrate that PAK4 is a target of KPT-9274. In a very recent paper, Neggers and colleagues used CRISPRres, “a CRISPR-Cas-based genetic screening approach to rapidly derive and identify drug resistance mutations in essential genes”, to identify the targets of KPT-9274. They showed that NAMPT is the principal target of this investigational compound^[162]. KPT-9274 remains the only agent in Phase I studies that target both PAK4 and NAMPT and KPT-9274 has demonstrated evaluable response in patients with solid tumor and hematological malignancies^[163,164]. Our laboratory has recently shown that KPT-9274 is effective against PNET models *in vitro* and *in vivo*^[139]. The drug blocks PAK4 signaling leading to inhibition of mTOR pathway molecules. We also demonstrated that KPT-9274 causes metabolic alterations in PNET cell that is reflective of its NAMPT targeted effects. More significantly, the drug synergized with everolimus and other commonly used therapies for PNETs. Based on these findings, it is anticipated that this agent will be evaluated in Phase 1b/II clinical study for advanced PNETs. The mechanism of action of KPT-9274 is illustrated in **Figure 3**.

CONCLUSION

The incidence of PNETs is vastly increasing worldwide; therefore, novel strategies to manage this specific neoplasia are urgently needed. Several factors contribute to the management failure of PNETs in the clinic. PNET is characterized by significant heterogeneity that is the major challenge associated with the management of this neoplasia. Also, the majority of PNET therapeutics only stabilizes the disease with minimal benefits for patients. Lack of specific biomarkers inhibits early diagnosis and the selection of effective drugs in the clinic. The absence of preclinical models, mainly cellular models, limits effective anticancer examination and a better understanding of the biology of PNETs in the laboratory. Immunotherapy does not work in this patient population. Nevertheless, several molecules are emerging as new therapeutic targets for the management of PNETs. FOXM1 that is involved in all the hallmarks of cancer has been identified as a new target to effectively manage tumorigenicity, growth and proliferation in gastroenteropancreatic neuroendocrine tumors. The matricellular proteins CYR61 has also been identified as tumor-promoting gene in PNETs. Finally, overexpression of PAK4 and NAMPT in PNET patients' biopsies suggests that inhibition of these two targets could become a feasible strategy for therapy resistant PNETs.

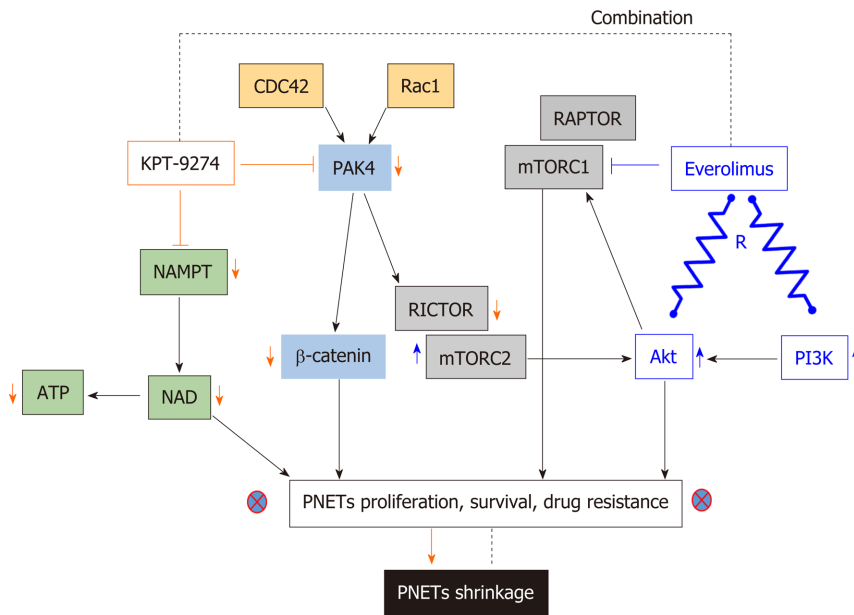


Figure 3 Mechanism of action of available PAK4-NAMPT dual inhibitor. Dual inhibition of PAK4 and NAMPT using KPT-9274 single agent or in combination with everolimus results in lower case tumor shrinkage. KPT-9274 inhibits NAMPT causing downregulation of NAD and ATP and alteration of cell metabolism. Additionally, KPT-9274 inhibits PAK4 resulting in downregulation of β -catenin and RICTOR. Downregulation of RICTOR causes the inhibition of mTORC2 implicated in everolimus resistance. R: Resistance; PAK4: p21-activated kinase 4; NAMPT: Nicotinamide Phosphoribosyltransferase; KPT-9274: Available PAK4-NAMPT dual inhibitor; NAD: Nicotine adenine dinucleotide; ATP: Adenosine-triphosphate; RICTOR: Rapamycin-insensitive companion of Tor; mTORC2: Mammalian target of rapamycin complex 2.

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Differential regulation of JAK/STAT-signaling in patients with ulcerative colitis and Crohn's disease

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Abstract

In 2018, the pan-Janus kinase (JAK) inhibitor tofacitinib was launched for the treatment of ulcerative colitis (UC). Although tofacitinib has proven efficacious in patients with active UC, it failed in patients with Crohn's disease (CD). This finding strongly hints at a different contribution of JAK signaling in both entities. Here, we review the current knowledge on the interplay between the JAK/signal transducer and activator of transcription (STAT) pathway and inflammatory bowel diseases (IBD). In particular, we provide a detailed overview of the differences and similarities of JAK/STAT-signaling in UC and CD, highlight the impact of the JAK/STAT pathway in experimental colitis models and summarize the published evidence on JAK/STAT-signaling in immune cells of IBD as well as the genetic association between the JAK/STAT pathway and IBD. Finally, we describe novel treatment strategies targeting JAK/STAT inhibition in UC and CD and comment on the limitations and challenges of the new drug class.

Key words: Janus kinase; Signal transducer and activator of transcription; JAK/STAT pathway; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; JAK/STAT inhibition; Tofacitinib

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Core tip: The pan-Janus kinase (JAK)-inhibitor tofacitinib is efficacious in patients with active ulcerative colitis (UC) but not Crohn's disease (CD), which hints at different contributions of JAK-signaling in both entities. In this review, available data on differential JAK/signal transducer and activator of transcription (STAT)-signaling in UC and CD were analyzed. The literature review identified differential cell-subset specific JAK/STAT-signaling including increased T-cell-associated STAT1 signaling in CD and STAT6 signaling in UC, while in myeloid cells inflammatory STAT1 was increased in UC compared with CD indicating a less inflammatory role of myeloid cells in CD. Development of JAK/STAT-inhibitors with specific targeting of associated inflammatory pathways might further improve the efficacy and safety profiles of this drug class.

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INTRODUCTION

Inflammatory bowel diseases (IBD) comprise the entities ulcerative colitis (UC) and Crohn's disease (CD) as well as unclassified IBD, which are chronic remitting diseases characterized by intestinal inflammation and the risk of uncontrolled disease activity which may lead to severe complications such as fistulas and strictures in CD, and colorectal neoplasia in both entities^[1-5]. Despite the plethora of available medical treatment options for IBD, treatment of patients is still complex and often challenging due to loss of response as well as adverse events including opportunistic infections^[6-8]. Population based studies report that 46% of patients with CD and 14% of patients with UC are still being treated with systemic corticosteroids for more than 6 months to achieve remission^[9,10].

The introduction of biological therapy has improved the spectrum of anti-inflammatory treatment. Nevertheless, the induction and maintenance of remission can still be challenging due to primary non response and secondary loss of response to biological therapy. Indeed, approximately one third of patients with CD and UC were classified as primary non-responders, and up to 50% of patients with IBD had a secondary loss of response to biological therapy or had to stop treatment due to severe side effects^[6,11]. Anti-tumor necrosis factor (TNF) therapy has the risk of immunogenicity including provocation of an immune response with occurrence of neutralizing antibodies towards anti-TNFα resulting in secondary loss of response. These observations clearly underline the need for new therapeutic agents with improvement of tolerability and long-term efficacy^[12].

Targeting cytokine signaling is already a proven therapeutic strategy as blockade of TNFα has been shown to have good efficacy in the treatment of IBD. However, aside from TNFα, a wide range of cytokines are crucially involved in maintaining intestinal inflammation and reveal a major role in the pathogenesis of IBD^[13]. Thus, modulation of several IBD-associated cytokines simultaneously appears to be a promising therapeutic target in IBD^[14-16]. Janus kinases (JAKs) mediate intracellular signaling of various cytokines and growth factors^[17-20].

The JAK/STAT pathway

JAKs are potent therapeutic targets and blockade can potentially interfere with more than 50 cytokines^[17,18]. JAKs are constitutively bound to cytokine receptors and are crucial in biological responses, mediating signals *via* signal transducers and activators of transcription (STATs)^[21-23]. Four different JAK molecules (JAK1, JAK2, JAK3 and tyrosine kinase (TYK) 2) and seven members of the STAT family (STAT1, 2, 3, 4, 5a, 5b, 6) are known^[18,24-27]. STAT5a and STAT5b represent two proteins with almost identical amino acids but are encoded by different genes^[28]. Binding of cytokines to their receptors mainly activates specific JAKs and subsequent STATs as final initiators of JAK signaling and can lead to specific cellular responses^[29-31]. The general mechanisms of JAK/STAT-signaling are summarized in **Figure 1**. Due to the fact that JAK/STAT-signaling is utilized by various cytokines, these pathways have become prominent targets for simultaneous inhibition of multiple pro-inflammatory cytokines^[32-34].

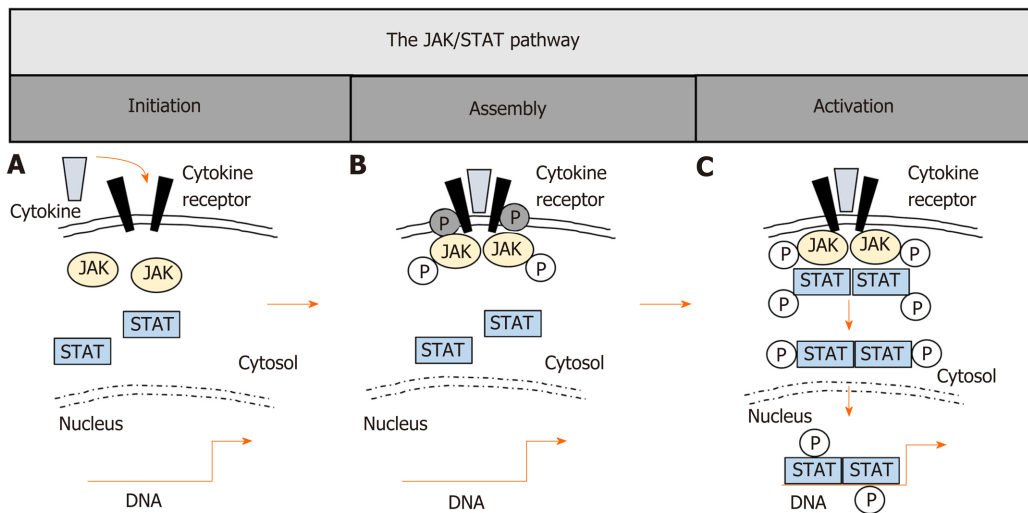


Figure 1 The JAK/STAT pathway. A: Canonical Janus kinase (JAK)/signal transducer and activator of transcription (STAT)-signaling initiates with the association of cytokines and their corresponding transmembrane receptors; B: Cytokine binding brings JAKs in proximity to the receptor, leading to phosphorylation of both the JAKs themselves and the cytoplasmic tails of the receptors, thereby creating docking sites for STAT monomers; C: STAT tyrosine phosphorylation (p-Tyr) is the major activating event, leading to dimerization of STATs, translocation to the nucleus, DNA binding of STAT dimers and subsequent target gene induction. JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

Nevertheless, targeting JAK/STAT-signaling is highly complex as overlapping JAK/STAT activation by various cytokines with induction of more than one specific downstream signaling pathway is known^[35-37]. In detail, similar JAK/STAT components can be activated by varying cytokines of related receptor families, which include cytokines of the interferon (IFN) receptor family *i.e.*, type I/II/III IFNs, IL-10, IL-19, IL-20, IL-22), the common γ -chain receptor family (*i.e.*, IL-2, IL-4 IL-7, IL-9, IL-15, IL-21), the gp130 receptor family (*i.e.*, IL-6, IL-11, IL-12, IL-23), the common β -chain receptors (*i.e.*, GM-CSF, IL-3, IL-5) and the single chain receptor family (*i.e.*, Epo, GH)^[29,30,38]. Besides these classical canonical pathways, which include subsequent JAK/STAT activation, non-canonical pathways with independent activation of either JAKs or STATs for signal transduction have been described^[39-41]. Furthermore, JAKs can also activate other downstream targets separate to the classical STATs^[42]. It is incompletely understood how specific signaling can be achieved regarding the complexity of JAK/STAT activation, but a cell subtype-associated specificity of JAK/STAT-signaling has been suggested^[31] as activation of the same STATs can lead to partially opposing effects in cells of the innate compared to the adaptive immune system. For instance, STAT3 mediates regulatory signaling in epithelial or myeloid cells^[43], while in lymphocytes, STAT3 activation results in predominantly pro-inflammatory responses such as Th17 differentiation and inhibition of regulatory T-cells^[44-46], which further underlines the great plasticity and complexity of JAK/STAT-signaling in regulatory or inflammatory responses of different cell compartments.

Clinical efficacy of JAK inhibition in RA and IBD

The JAK/STAT pathway has been associated with several immune-mediated diseases besides its important impact in cellular signaling^[47-50]. The pan-JAK inhibitor tofacitinib results in predominant inhibition of JAK1/JAK3 at adequate dosage^[51,52], leading to inhibited JAK-associated intracellular signaling of various cytokines and growth factors^[17,18] and was first developed and approved as a synthetic disease-modifying anti-rheumatic drug (DMARD)^[53-56] for the treatment of moderate to severe rheumatoid arthritis (RA) in 2017 throughout Europe^[53-55,57]. Due to its clinical efficacy in RA, tofacitinib was subsequently investigated as a treatment option for IBD^[58-61].

In a recently published phase III RCT, tofacitinib demonstrated efficacy for induction and maintenance of remission in patients with UC^[61], which led to its approval for treatment of moderate-to-severe UC^[62]. More specifically, 40.6% of patients treated with 10 mg tofacitinib twice daily achieved remission at week 52 as the primary endpoint as compared to 11.1% of patients treated with placebo^[61]. Of note, in patients with CD, tofacitinib did not reach its primary endpoints including clinical response or remission at week 26 of maintenance in a phase IIb clinical trial^[58]. In detail, clinical response or remission rates for treatment with 10 mg twice daily were

55.8% compared to 38.1% with placebo treatment, which was not significantly different. The lack of clinical response to tofacitinib treatment in patients with CD was further confirmed by the observation of worsening disease activity in 19.3% of patients treated with 10 mg twice daily in an open-label 48-week extension phase II clinical trial^[63] which led to premature termination of the trial. However, in the phase IIb trial, biomarkers of inflammation such as C-reactive protein were different in both cohorts with increasing levels in the placebo group and stable levels in the treatment group indicating a likely treatment effect of tofacitinib even in patients with CD^[58]. There is ongoing debate regarding the possibility of lack of therapeutic efficacy being explained by an unusually high placebo rate and the small sample size of the study. On the other hand, the lack of superiority of tofacitinib over placebo in patients with CD in clinical trials might also reflect different pathogenic roles of JAK/STAT-signaling in UC and CD^[64,65].

Previous data from our own group demonstrated that a more regulatory monocyte phenotype was induced by tofacitinib at adequate dosage under inflammatory conditions^[66]. In line with the observations from clinical trials, the regulatory impact of tofacitinib was stronger in monocytes derived from patients with UC as compared to CD^[66]. These observations led to the hypothesis that the JAK/STAT pathway might be differentially activated in UC, mediating stronger inflammatory responses compared to CD. Therefore, we summarize and discuss the available evidence on JAK/STAT-signaling in patients with UC and CD in different cell compartments as well as the genetic association and further discuss the clinical implications.

JAK/STAT-SIGNALING IN IBD: T-CELLS

The JAK/STAT pathway has a crucial impact on the regulation of T-cell differentiation. Additionally, dysregulated JAK/STAT-signaling leading to aberrant T-cell differentiation as well as defective regulatory T-cell activity has been suggested as important in IBD pathogenesis^[43,67-70]. Excessive T-cell activation and infiltration of the colonic mucosa has been observed in both CD and UC^[70,71], but the precise role of T-cells in both phenotypes is still a matter of debate. Historically, aberrant Th1 differentiation and associated cytokines (like IFN γ and IL-2) were predominantly associated with the pathogenesis of CD^[72] and Th2-cell-associated cytokines with UC^[73]. However, this concept has partially been abandoned as the contribution of different T-cell subsets infiltrating the intestinal mucosa is far more complex and has been demonstrated as a core player in the pathogenesis of both entities^[69,74,75]. Differences in the JAK/STAT-signaling in T-cells obtained from UC and CD have also been described, which may lead to similar but incongruent T-cell differentiation in both entities.

Most studies assess STAT-signaling as a surrogate marker for JAKs. Before we focus on IBD-related aberrant STAT-signaling in the T-cell compartment, we summarize the impact of JAK/STAT-signaling on T-cells in experimental colitis. Available data on T-cell attributed STAT expression/activation in UC and CD has also been summarized in [Figure 2](#).

Experimental colitis

T-cell-associated IFN γ induced a reduction in epithelial integrity and Paneth cells in mouse crypt enteroids, and led to exacerbation of radiation colitis^[76]. This effect could be abolished by JAK1,2 inhibition indicating the important impact of the JAK/STAT pathway in T-cell-mediated impairment of the intestinal barrier. Furthermore, several anti-inflammatory molecules ameliorating disease activity in experimental colitis are associated with modulation of JAK/STAT-signaling in lymphocytes. In detail, decreased expression or activation of JAK1 and JAK2^[77], STAT1^[77], STAT3^[77-79] and STAT4^[77,79] as well as enhancement of STAT5^[78] phosphorylation in T-cells, have been linked to effective pharmacological treatment of DSS-induced murine colitis and TNBS-induced rat colitis. Additionally, inflammatory activity was reduced by general STAT1 knockout in DSS-treated STAT1-null mice^[80] and by specific inhibition of STAT1 in T-cells in TNBS-induced colitis^[81]. Furthermore, knockdown of TYK2 ameliorated DSS-induced and TNBS-induced murine colitis possibly due to reduced Th1 and Th17 activity^[82]. In addition, in murine colitis, STAT6/Th2-linked cytokine IL-4 is associated with reduced Treg induction^[83,84]. In contrast to this, STAT6-deficient mice were shown to be protected against experimental colitis, partially due to reduced Th2 cytokines^[85].

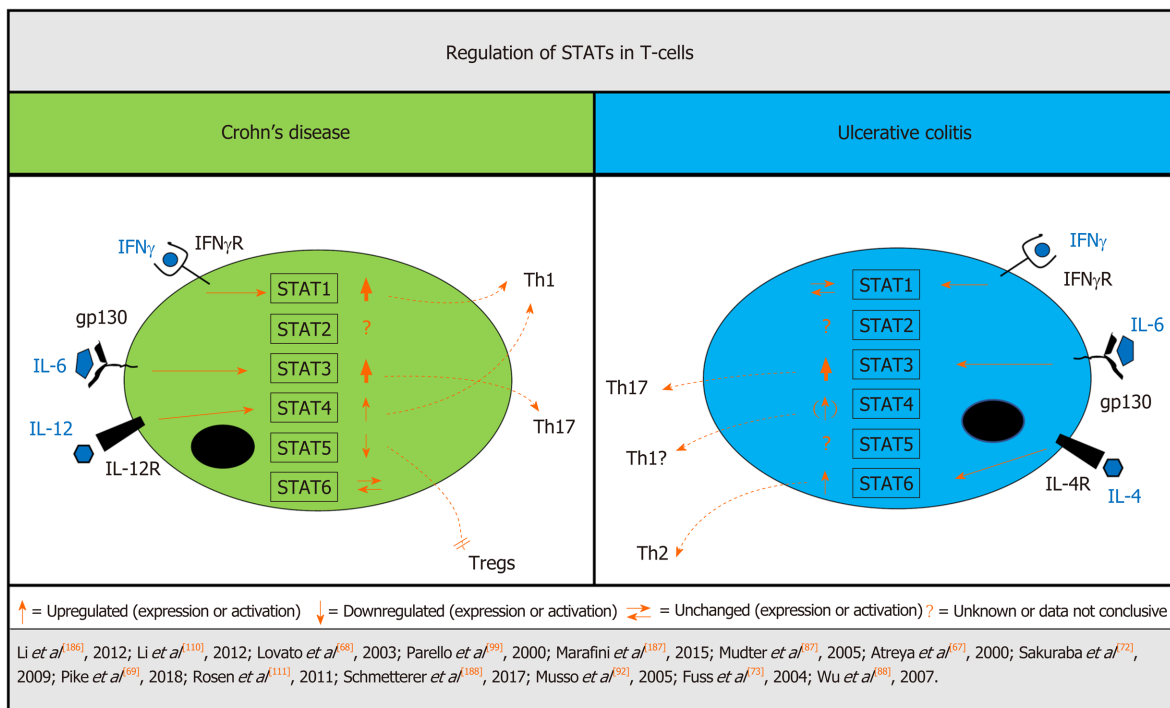


Figure 2 Differentially regulated STATs in T-cells from patients with inflammatory bowel disease. In T-cells, signal transducer and activator of transcription (STAT)1 signaling is increased in Crohn's disease (CD) but not ulcerative colitis (UC), while STAT3 is associated with a critical role in both UC and CD pathogenesis and overactivation is linked to increased intestinal inflammation. There is stronger evidence of STAT4 signaling in CD but STAT4 induction is also apparent in UC, while the STAT6 pathway seems to be more affected in UC. Down-regulation of STAT5 in CD leads to inhibition of regulatory T-cells. Strength of arrows indicates available supporting data. CD: Crohn's disease; IFN: Interferon; IFN γ -R: Interferon γ receptor; IL: Interleukin; IL4R: Interleukin 4 receptor; IL12R: Interleukin 12 receptor; STAT: Signal transducer and activator of transcription; Th: T-helper cell; Treg: Regulatory T-cell; UC: Ulcerative colitis.

STATs

STAT1 is closely linked to IFN γ -receptor signaling and has an important involvement in Th1 differentiation^[86]. Mudter *et al.*^[87] found increased IFN γ -induced STAT1 but not phospho-STAT1 in lamina propria T-cells of CD compared to patients with UC. Similarly, elevated gene expression of STAT1 as well as that of other IFN γ -Th1-related genes was detected by Wu *et al.*^[88] in colonic biopsies of patients with CD but not UC. Of note, the latter study did not provide cell specific investigations or analysis of STAT1 on protein level. Taken together, these data confirm an important role for STAT1-associated Th1 response in CD.

STAT2 is associated with type I IFN signaling^[89]. So far, only a trend towards decreased STAT2 gene expression in lamina propria mononuclear cells (LPMC) from patients with active UC and CD has been reported^[87] without further available data on STAT2 signaling in IBD.

In contrast, the impact of STAT3 in T-cell differentiation has been widely investigated. JAK1, JAK2 and TYK2 are involved in the activation of STAT3^[29] and increased STAT3 activation and signaling contributes to pathogenic Th17 differentiation and promotion of inflammation^[45], and is involved in the pathogenesis of UC and CD^[74,75]. However, STAT3 has also been linked to IL-10-dependent regulatory features of Tregs^[90,91] and the definitive role of STAT3 in T-cell regulation and its contribution to IBD has not been finally elucidated. Various studies on T-cell-associated STAT3 signaling have demonstrated increased levels of STAT3 and phospho-STAT3 in mucosal tissue samples from patients with UC and CD^[67,68,87,92]. Furthermore, Jin *et al.*^[93] performed transcriptomic and proteomic analyses of colonic biopsies and demonstrated that the inflammatory response of the IL-6-JAK/STAT3 signaling pathway was positively enriched in CD and UC samples. T-cells represent a large population of immune cells in the intestinal mucosa^[94] but whether these data could be attributed to only T-cells is not clear, as no cell compartment specific analysis was performed. Specific analyses of STAT3 signaling in CD-derived intestinal T-cells revealed constitutive STAT3 activation^[68] with IL-6-attributed induction of downstream anti-apoptotic genes^[67]. Furthermore, impaired STAT3-regulating mechanism has been identified in IBD. In a healthy environment, the phosphatase DUSP2 is induced by activation of T-cells and inhibits STAT3. Impaired DUSP2

expression is associated with enhanced STAT3 signaling and Th17 induction^[45]. It is noteworthy that DUSP2 has been demonstrated to be decreased in peripheral blood samples derived from patients with UC and active disease flare^[45]. However, no direct comparison with CD patients has been performed to date and no studies have investigated the role of DUSP2 in CD to date. Another STAT3-regulating factor is the protein tyrosine phosphatase PTPN2, which is linked to prevention of auto-reactivity in the context of T-cell homeostasis by STAT3 dephosphorylation^[95]. Deficiency of PTPN2 is associated with autoimmunity by increased lymphocyte activation^[96] and PTPN2 genetic risk locus with loss of function has been linked to CD^[95]. Taken together, impaired direct or indirect STAT3 regulation leading to increased STAT3 activation seems to be involved in both UC and CD pathogenesis, even though underlying mechanisms might partially differ.

The role of STAT4 in T-cells has been well described and various studies demonstrated an IL-12-triggered STAT4-dependent development of IFN γ -secreting Th1-cells^[97,98]. Available data on STAT4 in colonic tissue samples from patients with IBD are controversial; while increased IL-12-dependent expression and activation of STAT4 in T-cells from colonic mucosa was found in CD^[68] associated with IL-12-dependent Th1 polarization^[99], no increased T-cell-associated STAT4 signaling was detected in patients with UC^[99]. In contrast to these studies, increased STAT4 was found in the mucosa of adult^[100] and pediatric patients with UC but not in patients with CD^[101]. However, subtype analyses of intestinal cells were not performed in the latter studies, thus data can only speculatively be linked to STAT4 signaling in T-cells. In summary, published evidence of increased T-cell-associated STAT4 signaling in CD is strong, emphasizing the important role of Th1 response in CD pathogenesis. In UC, increased STAT4 signaling was also detected, but direct linkage to T-cells was not investigated: Thus, Th1 response may also play a distinct role in intestinal inflammation in UC but still remains a matter of debate.

STAT5 is mainly linked to induction of regulatory FoxP3⁺T-cells (Tregs)^[102] and limitation of Th17 differentiation *via* IL-2^[103], while Treg development is negatively controlled by STAT3^[104] and thus disruption of the STAT3/STAT5 balance might shift T-cell differentiation towards Th17 development^[46]. Tregs can limit autoimmunity, but in IBD, Tregs are not able to control inflammation due to increased induction and differentiation of effector T-cells^[105,106]. Direct data on STAT5 in IBD T-cells are scarce. In CD, $\alpha 4\beta 7^+CD4^+$ cells, which are strongly associated with gut-homing lymphocytes, show decreased induction of pSTAT5 in response to IL-2, while pSTAT3⁺ cells were increased after specific stimulation^[107]. However, data on T-cell-associated STAT5 signaling in UC are still missing.

STAT6 is associated with Th2 differentiation^[108,109]. Although Mudter *et al*^[87] found no increased STAT6 expression in LPMCs in IBD patients, there was increased activation of STAT6 in colonic tissue of inactive UC^[110] as well as specifically in LPMCs in UC, in contrast to lower levels in CD^[111]. It is noteworthy that T-cells represent the largest immune cell population of LPMCs; however, no specific T-cell analysis was performed. Nevertheless, both studies point to the overactivation of STAT6 in UC, which further underlines the importance of the Th2 response in UC pathogenesis, while in CD the role of STAT6 remains unclear.

JAK/STAT-SIGNALING IN IBD: MONOCYTES AND MONOCYTE-DERIVED CELLS

Monocytes are central to our health as uncontrolled and sustained inflammation can lead to auto-inflammatory syndromes and sometimes to autoimmune diseases. Monocytes can be a driving force in such diseases when their ability to also contribute to the resolution of inflammation is impaired. Therefore, anti-inflammatory mechanisms of monocytes, are of vast importance for downregulation and resolution of inflammation^[112-115]. As an example, we recently demonstrated that GM-CSF-activated monocytes also have regulatory capabilities such as in the induction of Tregs from naïve T-cells in *in vitro* co-cultures thus leading to amelioration of experimental colitis in mice *in vivo*^[116,117]. Furthermore, in IBD, monocytes are key players and function as effectors of inflammation^[118-120], presumably because most intestinal macrophages are replenished by peripheral monocytes from the circulation^[121,122]. In the intestine, recruited monocytes immediately adapt to the local environment. When they replenish intestinal monocytes during homeostasis, they become tolerogenic, but they differentiate into inflammatory drivers in the presence of intestinal inflammation^[123] which turns monocytes into important targets for treatment.

JAK/STAT-signaling does play decisive roles in monocyte-differentiation and regulation of monocyte-activation^[66,124]. For example, de Vries *et al*^[124] demonstrated that JAK1/STAT1 inhibition resulted in a prominent switch of macrophages from pro-inflammatory M1-like to a more regulatory M2-like phenotype, which leads to an enhanced recovery in acute rescue DSS-colitis. Similarly, JAK1/3 inhibition dose-dependently leads to a predominant M2-like phenotype shift of human monocytes derived from healthy volunteers and patients with IBD accompanied by an increased anti-inflammatory potential reflected by enhanced Treg induction^[66].

JAK/STAT

In monocytes, STAT1 activation *via i.e.*, IFN γ R is believed to be strongly inflammatory^[125,126]. Schreiber *et al*^[127] showed that patients with active UC had significantly higher levels of STAT1 expression and activation in colonic tissue compared to active CD, which could be predominantly attributed to infiltrating peripheral neutrophils and monocytes/macrophages. Among other regulatory mechanisms, STAT activation is inhibited by proteins of the suppressor of cytokine signaling (SOCS) family^[128-131] and SOCS3 and SOCS1 are involved in STAT1 regulation. Indeed, Schreiber and colleagues^[127] further detected that increased STAT1 activation was associated with distinctly lower mucosal SOCS3 protein levels in UC compared to patients with CD. In contrast, Soendergaard *et al*^[132] found SOCS1 and SOCS3 mucosal RNA level to be upregulated in an inflammation-dependent manner in UC compared to controls; however, no patients with CD were included in this study and direct comparison is missing.

Opposite to STAT1, STAT3 is associated with a more protective role in myeloid cells against inflammation and seems to be important for mucosal homeostasis^[43,133,134]. In their mouse study, Takeda *et al*^[43] genetically disrupted STAT3 in macrophages and neutrophils resulting in chronic enterocolitis induced by high levels of pro-inflammatory cytokines. Furthermore, the suppressive effects of IL-10 on macrophages and neutrophils were completely abolished in mice lacking STAT3 in these cells, further emphasizing the importance of STAT3 signaling in the regulation of inflammation^[43]. Depending on the environmental stimuli, monocytes, when leaving the bloodstream, can differentiate into macrophages and dendritic cells (DCs). An analysis of STAT activation in subsets of DCs from patients with CD revealed enhanced IL-10-induced STAT3-signaling in myeloid DCs^[135]. The study did not include patients with UC, so the role of STAT3 in UC-derived DCs remains unclear. Monocyte-specific expression of other STAT members including STAT2, STAT4, STAT5 and STAT6 has not yet been studied in IBD in detail. Available data on STAT expression/activation in monocytes and -derived cells in UC and CD are summarized in **Figure 3**.

Taken together, the contribution of JAK/STAT in IBD-derived myeloid cells in driving inflammation cannot be elucidated yet due to the scarcity of available data. However, STAT1 seems to be particularly increased in monocytes/macrophages in UC but not CD, which is possibly linked to an insufficient mechanism to regulate STAT1 signaling, and hints at a more pro-inflammatory phenotype of monocytes/macrophages in UC compared to CD.

GENETIC ASSOCIATION OF IBD AND THE JAK/STAT PATHWAY

For IBD, more than 250 genome susceptibility loci could be specified, of which some reveal an association with the JAK/STAT pathway^[136-139]. However, systematic analysis and comparison of the JAK/STAT components and associated genetic risk loci in UC and CD are scarce. We therefore summarize the available studies on JAK/STAT-associated susceptibility genes in IBD and elucidate the similarities and differences in UC *vs* CD in the next paragraphs and in **Table 1**^[136,138-158].

JAKs

To date, the genetic risk loci associated with JAK1 have not been identified in IBD. This appears reasonable, as JAK1-deficient mice are non-viable^[159,160], which underlines the fundamental involvement of JAK1 in cell signaling as essential for survival^[160,161]. Similarly, JAK3-linked genetic risk loci have not been detected in IBD so far. Patients suffering from genetic defects in JAK3 develop severe combined immunodeficiency without specificity for IBD^[161,162].

In contrast, an association of JAK2 gene variants with both UC and CD has been clearly shown in various studies^[136,138,140]. Especially the IBD risk locus rs10758669

Table 1 Genetic association between JAK/STAT-signaling and the development of inflammatory bowel disease

	Ulcerative colitis	Crohn's disease
JAK1	No association found to date	No association found to date
JAK2	Yang <i>et al</i> ^[140] , 2011	Yang <i>et al</i> ^[140] , 2011
	Barrett <i>et al</i> ^[136] , 2008	Barrett <i>et al</i> ^[136] , 2008
	Wellcome trust case control	Wellcome trust case control
	Consortium ^[138] , 2007	Consortium ^[138] , 2007
	Anderson <i>et al</i> ^[141] , 2009	Anderson <i>et al</i> ^[141] , 2009
	Zhang <i>et al</i> ^[142] , 2014	Zhang <i>et al</i> ^[142] , 2014
	Hedl <i>et al</i> ^[143] , 2016	Hedl <i>et al</i> ^[143] , 2016: N/A
	Cleynen <i>et al</i> ^[144] , 2013: N/A	Cleynen <i>et al</i> ^[144] , 2013
	Ferguson <i>et al</i> ^[145] , 2010: N/A	Ferguson <i>et al</i> ^[145] , 2010
	Prager <i>et al</i> ^[146] , 2014: No association	Prager <i>et al</i> ^[146] , 2014
JAK3	No association found to date	No association found to date
TYK2	Can <i>et al</i> ^[147] , 2015	Can <i>et al</i> ^[147] , 2015
	Lian <i>et al</i> ^[148] , 2013	Lian <i>et al</i> ^[148] , 2013: No association
	Sato <i>et al</i> ^[149] , 2009: No association	Sato <i>et al</i> ^[149] , 2009
STAT1	No association found to date	No association found to date
STAT2	No association found to date	No association found to date
STAT3	Barrett <i>et al</i> ^[136] , 2008	Barrett <i>et al</i> ^[136] , 2008
	Wellcome Trust Case Control	Wellcome Trust Case Control
	Consortium ^[138] , 2007	Consortium ^[138] , 2007
	Anderson <i>et al</i> ^[150] , 2011	Anderson <i>et al</i> ^[150] , 2011
	Willson <i>et al</i> ^[151] , 2012: N/A	Willson <i>et al</i> ^[151] , 2012 (pediatric patients)
	Zhang <i>et al</i> ^[142] , 2014	Zhang <i>et al</i> ^[142] , 2014
	Prager <i>et al</i> ^[146] , 2014: No association	Prager <i>et al</i> ^[146] , 2014
STAT4	Glas <i>et al</i> ^[152] , 2010: No association	Glas <i>et al</i> ^[152] , 2010
	Diaz-Gallo <i>et al</i> ^[153] , 2010	Diaz-Gallo <i>et al</i> ^[153] , 2010: No association
	Liu <i>et al</i> ^[139] , 2015	Liu <i>et al</i> ^[139] , 2015: No association
STAT5	Huang <i>et al</i> ^[154] , 2015 (STAT5A/STAT3 haplotypes): No association	Huang <i>et al</i> ^[154] , 2015 (STAT5A/STAT3 haplotypes)
STAT6	Klein <i>et al</i> ^[155] , 2015: No association	Klein <i>et al</i> ^[155] , 2015
	Xia <i>et al</i> ^[156] , 2003: No association	Xia <i>et al</i> ^[156] , 2003: No association
	Chua <i>et al</i> ^[157] , 2016: No association	Chua <i>et al</i> ^[157] , 2016: No association
	de Jong <i>et al</i> ^[158] , 2003: No association	de Jong <i>et al</i> ^[158] , 2003: No association

JAK: Janus kinase; N/A: Not investigated in this cohort; STAT: Signal transducer and activator of transcription.

within the JAK2 region has been widely investigated. Alongside associations with both UC and CD^[136,141,142], gain of function of this gene variant including increased JAK2 signaling in macrophages of patients with UC and healthy donors carrying the rs10758669 CC genotype was shown^[143]. Furthermore, in CD, the JAK2 variant rs10758669 was associated with a more complicated disease course and shorter time interval to stenosis occurrence^[144,145]. Increased intestinal permeability was detected in patients with CD with the C risk allele within this JAK2 variant, which might hint at JAK2 being involved in increasing permeability in IBD as a possible pathomechanism^[146].

The available data of TYK2 gene polymorphism and IBD association are rare and

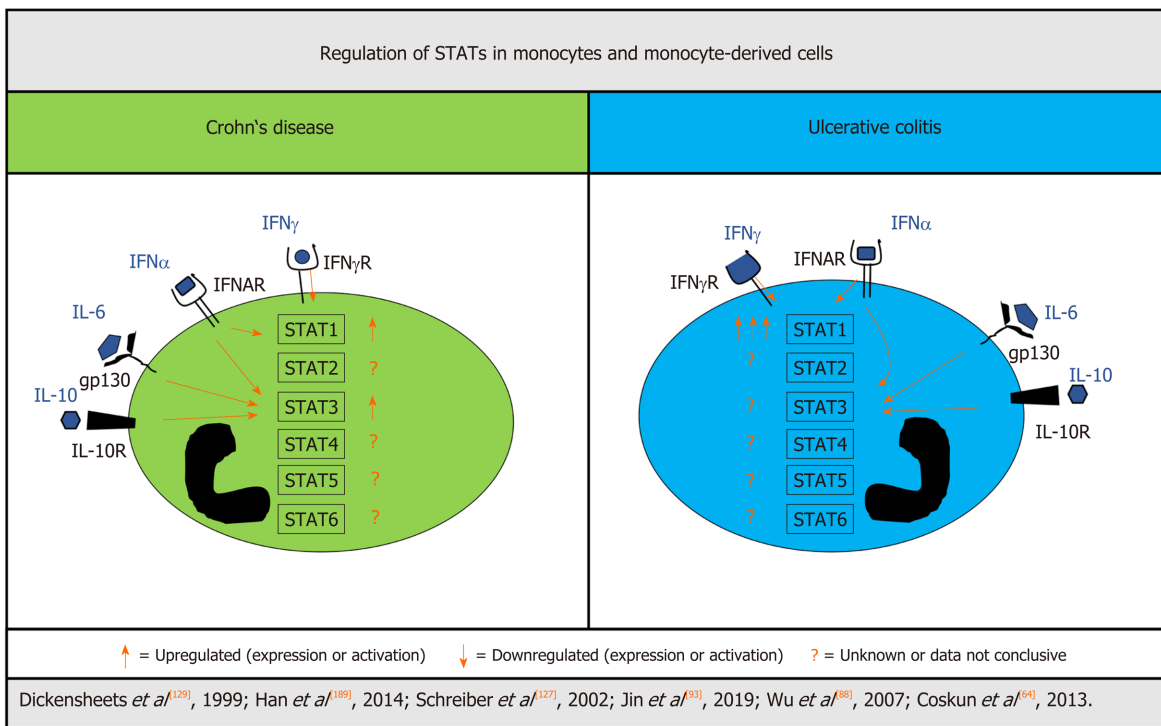


Figure 3 Differentially regulated STATs in monocytes and monocyte-derived cells from patients with inflammatory bowel disease. Signal transducer and activator of transcription (STAT)1 activation seems to be different in myeloid cells of patients with Crohn's disease (CD) and ulcerative colitis (UC). Although induced in CD, STAT1 is greatly elevated in monocytes and monocyte-derived cells from UC. There is evidence for increased STAT3 signaling in CD, while UC was not investigated. For STAT2, STAT4, STAT5, and STAT6 there are no solid data available from patients with IBD. Count of arrows indicates strength of increase in a direct comparison of UC and CD. CD: Crohn's disease; IFN: Interferon; IFNAR: Interferon α receptor; IFN γ -R: Interferon γ receptor; IL: Interleukin; IL10R: Interleukin 10 receptor; STAT: Signal transducer and activator of transcription; UC: Ulcerative colitis.

divergent. Although TYK2 has been identified as a susceptibility gene for both UC and CD in the Turkish population^[147], in Malaysian patients no association between CD and TYK2 genes has been detected^[148]. Conversely, in the Japanese population, a strong association of TYK2 with CD, but not UC was found^[149].

STATs

Contrary to STAT1/STAT2, for which no genetic association for UC or CD has been found to date, STAT3 gene variants have been distinctly associated with susceptibility for both, CD and UC^[136,138,150,163]. Willson *et al*^[151] analyzed STAT3 genetic variant rs744166 and demonstrated that pediatric patients with CD carrying STAT3 "A" risk allele revealed enhanced STAT3 activation in intestinal tissue and increased signaling linked to intestinal leukocyte homing. A meta-analysis including 10298 patients with CD and 4244 patients with UC, which further evaluated this STAT3 variant, confirmed the (A) allele to increase susceptibility for both UC and CD^[164]. Of note, Caucasian carriers of the (A) allele were more susceptible to UC and CD as compared to other ethnicities^[164].

Available data on STAT4 genetic variants linked with UC or CD are divergent. Jostins *et al*^[137] detected an increased association of STAT4 polymorphisms with IBD in general without specific association with either UC or CD. Glas *et al*^[152] investigated STAT4 genetic variant rs7574865 and found an association with colonic disease manifestation and early onset of disease in CD without increased UC susceptibility. Conversely, two studies identified an association of rs7574865 with increased risk for UC but not CD^[139,153].

Specific polymorphisms in the STAT5 gene locus have not been detected for IBD to date. However, Huang and colleagues^[154] identified that STAT5A/STAT3 haplotypes were generally linked to IBD and to CD.

The data on STAT6 genetic variants and IBD susceptibility are also rare: In a small study, comprising 243 patients with CD and 100 patients with UC, Klein *et al*^[155] detected a link between STAT6 gene risk locus G2964A (rs324015) and the subgroup of patients with CD, which revealed no variation in the CARD15 gene. However, the association of CD with STAT6 polymorphism rs324015 could not be confirmed by other studies^[156-158], which suggests that STAT6 gene variants may not have an

important involvement in IBD susceptibility.

JAK/STAT-associated signaling and regulation

A distinct genetic association has been shown for specific protein tyrosine phosphatases (PTPs) which are involved in regulation of JAK/STAT-signaling. Among those, PTPN22, which is linked to the regulation of STAT1 and Th1^[69,165-167], has been detected as a risk gene for CD susceptibility without an association to UC^[137,150]. Surprisingly, PTPN22 variant rs33996649 was identified as protective against CD, while it is associated with an increased risk for other autoimmune diseases^[136,153,168]. Similarly, genetic variants of PTPN2, which is associated with regulation of STAT1 and STAT3 phosphorylation, were found to be linked to CD but not UC^[138]. In detail, patients with CD carrying PTPN2 loss-of-function variant rs1893217 showed increased Th1- and Th17-related transcription factors and cytokines. Similarly, STAT1/3 activation, which is associated with Th1 and Th17 differentiation, was increased in PTPN2-/-CD4 T-cells^[95]. However, in a large meta-analysis, associations between PTPN2 gene polymorphisms and both CD and UC were found, which emphasizes the importance of PTPN2 and associated regulation of STAT1/3-linked Th1 and Th17 differentiation in both entities^[169]. For PTPN11, regulating STAT1 signaling^[81,170], a genetic association with UC but not CD was found^[171]. Furthermore, in DSS-induced colitis, conditional PTPN11 knockout in T-cells increased colitis severity^[172] stressing the important role of PTPN11 in colitis pathogenesis. Additionally, several studies have identified a marked association between the cytokine receptor IL-23R and IL-12R genes, which are both linked to Th17 differentiation, in both UC and CD^[137,141,150,173]. The relevance of IL-23 and IL-12 signaling on IBD pathogenesis is further confirmed by the therapeutic efficacy of the IL-12/IL-23 blocker ustekinumab in both entities.

CLINICAL IMPLICATIONS

Regarding the summarized data, it becomes evident that aberrant signaling of various direct and indirect components of the JAK/STAT pathway are critically involved in initiation and/or perpetuation of inflammation in both UC and CD. Dysregulated JAK/STAT-signaling is possibly linked to specific JAK/STAT-associated genetic predispositions and is partially cell subset specific with different signaling aberrations in the innate *vs* adaptive immunity. With the regulatory drug approval of the pan-JAK inhibitor tofacitinib, therapeutic inhibition of the JAK/STAT pathway is available for the clinical management of patients with UC. While the OCTAVE study program has proven the efficacy of tofacitinib for UC, some questions regarding its safety in terms of herpes zoster infection^[61] and pulmonary embolism have occurred recently as severe adverse events in patients treated twice daily with 10 mg tofacitinib were observed^[174]. Furthermore, tofacitinib revealed no effective response in CD compared to placebo in a phase II RCT^[58]. These data underline that adequate selection of patients with UC for tofacitinib treatment is key.

Selective JAK/STAT-inhibition

Targeting JAK/STAT components more precisely rather than inhibiting the complete pathway appears promising to limit adverse events and potentially improve clinical response in CD. Various compounds associated with selective JAK inhibition, including filgotinib and upadacitinib as predominantly JAK1-selective inhibitors, are currently under clinical investigation for IBD, while upadacitinib has most recently been approved for the treatment of RA^[175,176]. Filgotinib has entered a phase III RCT due to promising results in moderate-to-severe CD including induction of clinical remission and mucosal healing^[177]. Results from the phase II RCT with upadacitinib also show dose-dependent favorable outcomes with 27% clinical remission rates in patients with CD treated with 6 mg twice daily^[178] and phase III studies in CD and UC are ongoing (NCT03006068, NCT03345836). Furthermore, the JAK3-selective inhibitor Pf-06651600 and dual JAK1/TYK2 inhibitor Pf-06700841 are currently being investigated in phase II RCTs for CD (NCT03395184) and UC (NCT02958865). Additionally, the agent BMS-986165 is a selective TYK2 inhibitor leading to blockade of IL-12-, IL-23-, and type I IFN signaling^[179] and is being investigated in moderate-to-severe CD in a phase II RCT (NCT03934216). The intestinally restricted pan-JAK inhibitor TD-1473 is also under investigation in a phase II RCT in CD (NCT03635112) and a phase II study is planned for UC (NCT03920254).

However, since all JAKs are involved in complex biological processes, which control a wide range of cellular responses, inhibition of a specific JAK may lead to imprecise

outcomes including both inhibition of regulatory and inflammatory pathways as well as unwanted side effects. Thus, specific inhibition of STATs as downstream effectors of JAK/STAT-signaling might be the next step in using the JAK/STAT pathway as a therapeutic target. However, therapeutic agents investigated in IBD still target JAKs and to our knowledge, no STAT inhibitor has been established for IBD treatment to date, which might be due to certain challenges: STATs lack catalytic activity and pharmacological targeting is complex compared to the kinase domains of JAK, which represent distinct targets for therapeutic inhibition^[31]. Nevertheless, STAT inhibitors are currently under investigation in several oncologic diseases such as acute myeloid leukemia or recurrent malignant glioma^[180]; thus, the investigation of pharmacological STAT targeting might also be prospectively possible in IBD.

Cell specific targeting

Of note, in considering JAK/STAT-signaling as a therapeutic target, one should take into account that STATs are associated with partially counter-directional functions in different cell compartments. Namely, STAT3 has different function in monocytes and monocyte-derived cells (innate immune system) with rather regulatory features *i.e.*, IL-10 release, while in T-cells (adaptive immune system) STAT3 is strongly pro-inflammatory involved in Th17 differentiation. It needs to be emphasized that an important regulatory impact of the innate immune system was suggested for CD^[143]. This is supported by the fact that in myeloid cells of patients with CD, STAT3 reveals strong activation^[135], while pro-inflammatory STAT1 is significantly lower in its expression and activation in myeloid cells of intestinal tissue of active CD compared to UC^[127]. Furthermore, our own observations demonstrate higher levels of pro-inflammatory cytokines including TNF α and IL-6 in a pro-inflammatory setting and a stronger susceptibility to regulatory tofacitinib treatment in UC compared to CD-derived monocytes^[66]. These data underline the suggestion of myeloid cells as part of the innate immune system which possibly plays a less inflammatory and more regulatory role in CD compared to UC. The pan-JAK inhibitor tofacitinib revealed no significant efficacy in CD and a possible explanation might be the broad JAK/STAT inhibition which targets T-cell associated inflammatory signaling^[17] but might simultaneously inhibit important regulatory responses in myeloid cells in CD. Indeed, the impact of JAK inhibition specifically on myeloid cells is controversial: Some studies describe an effective inhibition of pro-inflammatory responses by JAK blockade^[17,181], while conversely, upregulated pro-inflammatory signaling has also been described^[182,183]. During intestinal injury, peripheral monocyte recruitment to intestinal tissues is increased, which are pro-inflammatory and primed in an inflammatory environment to contribute to inflammatory outcomes^[66,184,185]. Thus, induction of pro-inflammatory myeloid cells by broad JAK inhibition might contribute to its sustained impact on intestinal inflammation. With regard to that, cell subset-specific JAK/STAT targeting has been suggested as a future therapeutic strategy in IBD to improve efficacy^[143], especially in CD.

Consideration of different genotypes

Last but not least, data on JAK/STAT components as genetic risk loci clearly indicate that patients with IBD reveal specific overlap and differences such as JAK2, STAT3 as common and STAT regulating PTP22 and PTPN11 as potential different genetic risk loci. In terms of an individualized therapy, genotypic adaptation of medical treatment or individualized drug dosing, which is already established for thiopurine treatment in patients with IBD by assessing TMPT genotypes, would be an elegant future clinical target for different JAK/STAT-associated genotypes. Hedl and coworker^[143] already demonstrated that monocyte-derived macrophages (MDMs) of patients with the JAK2 rs10758669 CC risk gene revealed enhanced JAK2 signaling and increased pro- and anti-inflammatory cytokines. In rs10758669 AA disease carrier-derived MDMs compared to C carrier-derived MDMs, lower doses of JAK2 inhibitor and tofacitinib changed decreasing to increasing inflammatory cytokines. These data further underline the need to adapt dosage or treatment to JAK-associated genotype. In addition to an improved individualized therapy, JAK-genotyping could be of use to anticipate disease course as was already shown for the JAK2 genetic variant rs10758669 and STAT4 genetic variant rs7574865, which were both associated with a complicated disease course in CD^[144,145,152].

SUMMARY

A clear assignment of JAK/STAT components to CD or UC pathogenesis is difficult due to pleiotropic and partially overlapping dysregulation of JAK/STAT molecules or its regulators. Nevertheless, some general conclusions can be drawn:

First, in T-cells, STAT3 is associated with a critical role in both UC and CD pathogenesis and over-activation is linked to increased Th17 response and intestinal inflammation. Aberrant T-cell linked STAT1 expression is predominantly detected in CD and STAT6 expression predominantly in UC, while there is evidence for dysregulation of STAT4 in both entities. These data indicate a partial confirmation of the Th1/CD and Th2/UC concept but also hint at a certain impact of Th1 in UC.

Secondly, the overlap of both UC and CD-associated risk genes STAT3, JAK2, IL23R and IL12R, all being involved in Th17-cell differentiation, further strongly underlines the contribution of dysregulated Th17 response to both UC and CD. Furthermore, risk loci of genes associated with Th1 differentiation were found in both UC and CD such as STAT4 in both entities and STAT1-regulating PTP22 in CD and PTP11 in UC, respectively. These data further underline the importance of Th1 response in CD but again emphasize an overlap of Th1-cells which also contributes to UC and weaken the hypothesis of the specific role of Th1 in CD.

Thirdly, in contrast to lymphocytes, the JAK/STAT pathway has a more regulatory impact on myeloid cells in IBD. Only STAT1, mainly increased in UC-derived myeloid cells, seems to be associated with rather inflammatory features which points to a more inflammatory role of the JAK/STAT pathway in UC than CD and might partially explain the clinical response to tofacitinib in UC compared to patients with CD.

CONCLUSION

To conclude, the summarized data strongly indicate that development of sophisticated JAK/STAT inhibitors might further improve the efficacy and safety profiles of this drug class and improve their positioning in the therapeutic algorithm. This might be achieved by specific targeting of JAK/STAT components or cell subset specific JAK/STAT inhibition in T-cells to minimize the effects of JAK/STAT blockade on myeloid cells. Finally, assessment of specific genotypes as part of a personalized treatment approach might not only increase safety and efficacy of JAK/STAT inhibition including optimized dosing, but could also serve as a predictive marker for disease course in CD.

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Helicobacter pylori infection: Beyond gastric manifestations

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Abstract

Helicobacter pylori (*H. pylori*) is a bacterium that infects more than a half of world's population. Although it is mainly related to the development of gastroduodenal diseases, several studies have shown that such infection may also influence the development and severity of various extragastric diseases. According to the current evidence, whereas this bacterium is a risk factor for some of these manifestations, it might play a protective role in other pathological conditions. In that context, when considered the gastrointestinal tract, *H. pylori* positivity have been related to Inflammatory Bowel Disease, Gastroesophageal Reflux Disease, Non-Alcoholic Fatty Liver Disease, Hepatic Carcinoma, Cholelithiasis, and Cholecystitis. Moreover, lower serum levels of iron and vitamin B12 have been found in patients with *H. pylori* infection, leading to the emergence of anemias in a portion of them. With regards to neurological manifestations, a growing number of studies have associated that bacterium with multiple sclerosis, Alzheimer's disease, Parkinson's disease, and Guillain-Barré syndrome. Interestingly, the risk of developing cardiovascular disorders, such as atherosclerosis, is also influenced by the infection. Besides that, the *H. pylori*-associated inflammation may also lead to increased insulin resistance, leading to a higher risk of diabetes mellitus among infected individuals. Finally, the occurrence of dermatological and ophthalmic disorders have also been related to that microorganism. In this sense, this minireview aims to gather the main studies associating *H. pylori* infection with extragastric conditions, and also to explore the main mechanisms that may

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explain the role of *H. pylori* in those diseases.

Key words: *Helicobacter pylori*; Extragastric; Neurological; Cardiovascular; Autoimmune; Ophthalmic; Diabetes; Timeline; Treatment

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Core tip: *Helicobacter pylori* is a bacterium that is known to infect the gastric environment and to be related to gastroduodenal diseases, including peptic ulcer and gastric adenocarcinoma. However, since the 80s the relationship between this infection and manifestations that affect not only the gastric system has been studied, such as inflammatory bowel disease, iron and B12 deficiency, non-alcoholic fatty liver disease, hepatic carcinoma, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and Guillain-Barré syndrome. In this sense, this study made a survey of these manifestations and their physiopathology.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that inhabits the gastric environment of 60.3% of the world population, and its prevalence is particularly high in countries with inferior socioeconomic conditions, exceeding 80% in some regions of the globe^[1]. This phenomenon occurs, among other reasons, due to the unsatisfactory basic sanitation and high people agglomerations observed in many underdeveloped nations, scenarios that favour the oral-oral and fecal-oral transmissions of *H. pylori*^[2]. Another possible transmission route of this pathogen currently being discussed is the sexual route^[3], since people with *H. pylori*-positive sexual partners have higher infection rates than control groups. It is well established that this microorganism is mainly related to the development of gastroduodenal disturbances, of which stand out peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma^[4-6]. However, since the 1980s, growing evidence have associated such infection with several extragastric manifestations (Figure 1)^[7].

In that context, *H. pylori* infection seems to influence the onset and the severity of diseases from multiple organ systems, behaving as a risk factor for a number of disorders but also as a protective agent against some conditions^[8]. Regarding the main diseases that affect organs other than the stomach in the gastrointestinal tract (GIT), the *H. pylori* infection appears to be associated with inflammatory bowel disease (IBD), gastroesophageal reflux disease (GERD), non-alcoholic fatty liver disease (NAFLD), hepatic carcinoma, cholelithiasis, and cholecystitis^[7]. Besides that, serum vitamin B12 and iron deficiencies are known to be worsen or even caused by *H. pylori* infection. In addition, ocular, dermatological, metabolic, cardiovascular, and neurological diseases are also related to that microorganism^[8,9].

Given the background, this minireview aimed to compile evidence supporting the main associations between *H. pylori* infection and extragastric diseases (Figure 2), as well as to gather information on the supposed mechanisms that may link that bacterium to manifestations occurring in organs far from their primary infection site (Table 1)^[10]. The publications with the highest level of evidence found for each non-gastroduodenal manifestation were selected and listed at Table 2.

EXTRAGASTRIC MANIFESTATIONS

IBD

One of the most studied conditions in gastroenterology field, IBD is a set of chronic disorders that affects the digestive tract and includes Crohn's disease (CD) and

Table 1 Non-gastric manifestations of *Helicobacter pylori* and their suggested mechanisms of pathophysiology

Non-gastric manifestation	Mechanisms of pathology suggested to be correlated
Allergic diseases	Hygiene hypothesis ^[9,96]
Alzheimer's disease	Vitamin B12 deficiency leading to increased concentrations of homocysteine ^[109] Anormal hyperphosphorylation of the TAU protein caused by <i>H. pylori</i> infection ^[109] ApoE polymorphism ^[110]
Asthma	Treg pattern, suppressing Th-2-mediated allergic response ^[94]
Atherosclerosis and myocardial infarction	Stimulation of foam production inside macrophages, contributing to the magnification of the atherosclerotic plaque and arterial dysfunction ^[122]
B12 deficiency	Still to be clarified, but proven to be independent of gastric atrophy and bleeding that impair their dietary absorption ^[49]
Cholelithiasis	Presence of <i>H. pylori</i> infected bile ^[43,44]
Coronary arterial disease/systemic arterial stiffness	Increased levels of homocysteine ^[132] .
Gastroesophageal reflux disease	Hyperacidity ^[25]
Diabetes mellitus	Increased cytokine production; phosphorylation of serine residues from the insulin receptor substrate ^[136]
Hepatic carcinoma	Inflammatory, fibrotic and, consequently, necrotic process ^[37,38]
Idiopathic thrombocytopenic purpura (ITP)	CagA may stimulate the synthesis of anti-CagA antibodies that cross-react with platelet surface antigens causing ITP ^[74,75]
Inflammatory bowel disease	Reduced intestinal inflammation through release of IL-18 and development of FoxP3-positive regulatory T cells ^[16-18] Neutrophil-activating protein reducing inflammation through Toll-like receptor 2 and IL-10 stimulation ^[19,20]
Iron deficiency anemia	Still to be clarified, but proven to be independent of gastric atrophy and bleeding that impair their dietary absorption ^[49] Relationship with growth disorders in children ^[52,53]
Multiple sclerosis	Hygiene hypothesis ^[9] Inhibitory induction of <i>H. pylori</i> over the Th1 and Th17 immune response ^[103]
Non-alcoholic fatty liver disease	<i>H. pylori</i> induced insulin resistance ^[32] Reduced production of adiponectin ^[33] Liver inflammation ^[34,35]
Ophthalmic manifestations	Systemic inflammatory status; increased oxidative stress; mitochondrial dysfunction; damage to DNA ^[82]
Parkinson's disease	Increased synthesis of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ^[118] Reduced levodopa absorption ^[118]

H. pylori: *Helicobacter pylori*; CagA: Cytotoxin-associated gene A.

ulcerative colitis (UC)^[111]. Although the mechanisms involved in IBD genesis are broadly studied, they are not well understood and may include genetic, immune, and environmental interactions^[12]. Among these complex interplays, the association between microorganisms and IBD has been broadly explored, and, interestingly, researches have pointed to a protective role of *H. pylori* gastric infection in that condition. In this sense, a meta-analysis that included 60 studies found a negative association between that infection and IBD (OR = 0.43, 95%CI: 0.36-0.50, $P < 1^{-10}$). Besides that, such protection relationship was stronger in CD (OR = 0.38, 95%CI: 0.31-0.47, $P < 1^{-10}$) and in IBD unclassified (OR = 0.43, 95%CI: 0.23-0.80, $P = 0.008$) when compared to UC (OR = 0.53, 95%CI: 0.44-0.65, $P < 1^{-10}$)^[13]. In addition, a cohort carried out in Taiwan observed an increased risk of IBD development after bacterial eradication (adjusted hazard risk = 2.15; 95%CI: 1.88-2.46, $P < 0.001$)^[14]. Furthermore, *H. pylori* infection seems not only reduces the risk of IBD acquirement but also seems to minimize the clinical severity of the disease. A recent study that evaluated CD

Table 2 Levels of evidence of the risk relationship between *Helicobacter pylori* infection and each non-gastroduodenal manifestation

Manifestation	Year of publication ¹	Ref. ¹	Level of evidence
Alopecia areata	2017	Behrangi <i>et al</i> ^[72]	III
Alzheimer's disease	2016	Shindler-Itskovitch <i>et al</i> ^[107]	II
	2020	Fu <i>et al</i> ^[108]	II
Arterial hypertension	2018	Wan <i>et al</i> ^[127]	III
Asthma	2013	Wang <i>et al</i> ^[90]	II
	2017	Chen <i>et al</i> ^[91]	III
Atherosclerosis	2019	Iwai <i>et al</i> ^[124]	III
B12 deficiency	2000	Kaptan <i>et al</i> ^[47]	I
	2018	Mwafy <i>et al</i> ^[48]	III
Central serous chorioretinopathy	2006	Cotticelli <i>et al</i> ^[88]	IV
Cholecystitis and cholelithiasis	2015	Guraya <i>et al</i> ^[43]	II
	2018	Tsuchiya <i>et al</i> ^[41]	III
	2018	Cen <i>et al</i> ^[44]	III
Coronary artery disease	2016	Sun <i>et al</i> ^[131]	II
Diabetes mellitus	2019	Chen <i>et al</i> ^[135]	III
Gastroesophageal reflux disease	2016	Wang <i>et al</i> ^[26]	II
Glaucoma	2018	Zeng <i>et al</i> ^[83]	III
	2002	Kountouras <i>et al</i> ^[84]	III
Guillain-Barré syndrome	2020	Dardiotis <i>et al</i> ^[120]	III
Halitosis	2017	HajiFattahi <i>et al</i> ^[29]	III
	2019	Anbari <i>et al</i> ^[30]	III
Hepatic carcinoma	2017	Huang <i>et al</i> ^[39]	III
Idiopathic thrombocytopenic purpura	2018	Kim <i>et al</i> ^[78]	II
Inflammatory bowel disease	2017	Castaño-Rodríguez <i>et al</i> ^[13]	III
	2019	Lin <i>et al</i> ^[14]	III
Iron deficiency anemia	2018	Mwafy <i>et al</i> ^[48]	III
Myocardial infarction	2015	Liu <i>et al</i> ^[125]	III
Multiple sclerosis	2007	Li <i>et al</i> ^[100]	III
	2016	Jaruvongvanich <i>et al</i> ^[101]	III
	2016	Yao <i>et al</i> ^[102]	III
Non-alcoholic fatty liver disease	2019	Liu <i>et al</i> ^[36]	II
Parkinson's disease	2020	Wang <i>et al</i> ^[118]	III
Psoriasis	2019	Yu <i>et al</i> ^[67]	II
	2017	Mesquita <i>et al</i> ^[64]	III
Rosacea	2017	Saleh P <i>et al</i> ^[59]	III
	2017	Jørgensen <i>et al</i> ^[62]	III

Adapted from the American Society of Plastic Surgeons rating scale for risk studies, 2011^[137].

¹Publications with the higher level of evidence found for the risk relationship between *Helicobacter pylori* infection and each non-gastroduodenal manifestation. Levels of evidence: I - High-quality, multi-centered or single-centered, prospective cohort or comparative study with adequate power, or a systematic review of these studies; II - Lesser-quality prospective cohort or comparative study, retrospective cohort or comparative study, untreated controls from a randomized controlled trial, or a systematic review of these studies; III - Case-control study, or systematic review of these studies; IV - Case

series with pre/post test, or only post test; V - Expert opinion developed *via* consensus process; case report or clinical example; or evidence based on physiology, bench research or “first principles”.

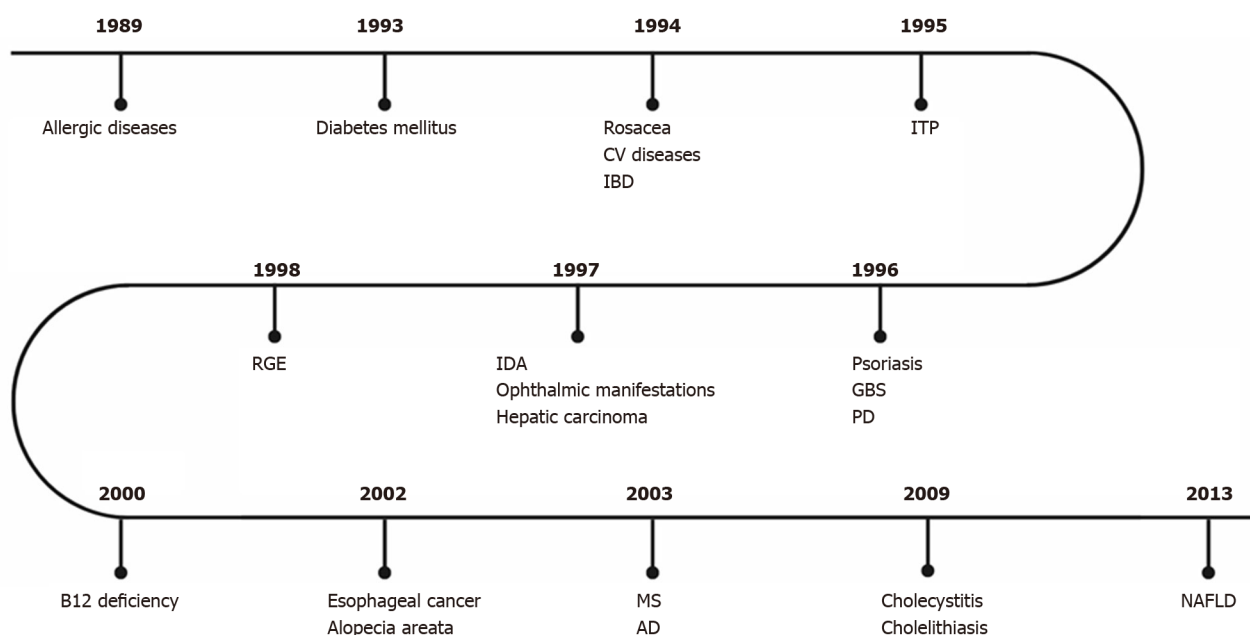


Figure 1 First studies on the association between *Helicobacter pylori* infection and extragastric manifestations over time. CV: Cardiovascular; IBD: Intestinal bowel disease; ITP: Idiopathic thrombocytopenic purpura; GBS: Guillain-Barré Syndrome; IDA: Iron deficiency anemia; RGE: Gastroesophageal reflux disease; PD: Parkinson's disease; MS: Multiple sclerosis; AD: Alzheimer's disease; NAFLD: Non-alcoholic fatty liver disease.

patients observed that *H. pylori* infection was negatively associated with fistulizing or stricturing phenotype (OR = 0.22, 95%CI: 0.06-0.97, $P = 0.022$), as well as with active colitis (OR = 0.186, 95%CI: 0.05-0.65, $P = 0.010$)^[15].

A hypothesis that can justify these findings is the fact that such infection induces interleukin (IL)-18 release, leading to the development of FoxP3-positive regulatory T cells, as well as decreases the maturation of antigen-presenting cells, what reduces intestinal inflammation^[16-18]. Another contributory mechanism may be the presence of the *H. pylori* neutrophil-activating protein that attenuate inflammation by means of the activation of toll-like receptor 2 and stimulation of IL-10 production^[19,20]. Finally, the composition of gut microbiota, which seems to play a crucial role in IBD development^[21], is significantly affected by the *H. pylori* eradication^[22]. In this sense, it is plausible to think that the changes in the intestinal microbiome may be decisive in the IBD onset after *H. pylori* treatment, although studies evaluating this proposition are not yet available.

GERD

Still regarding gastrointestinal diseases, GERD is characterized by the abnormal stomach content reflux through the esophagus, leading to damages in its organ mucosa, among other outcomes^[23]. Pyrosis, regurgitation, sore throat, cough, chest pain, and dysphagia are the most common symptoms in that condition^[24].

The role of *H. pylori* infection in GERD is controversial since its associated gastritis can lead both to an increase or to a reduction of acidic secretion, depending on the affected gastric region. On one hand, the *H. pylori*-associated antral gastritis causes hyperacidity, aggravating GERD. On the other hand, the corpus gastritis results in hypoacidity and plays a protective role against that disease. Such a protective behavior can be explained by bacterial genetic factors that influence *H. pylori* cytotoxin-associated gene A (CagA) positivity, once CagA-positive strains are associated with corpus atrophic gastritis and acidic secretion inhibition, which suggests that they may provide GERD protection^[25]. A meta-analysis conducted by Wang *et al*^[26], involving twenty randomized controlled trials, evaluated the onset of GERD-associated symptoms and esophageal lesions, comparing *H. pylori*-positive patients who underwent bacterial eradication with others who did not went through it. Their results

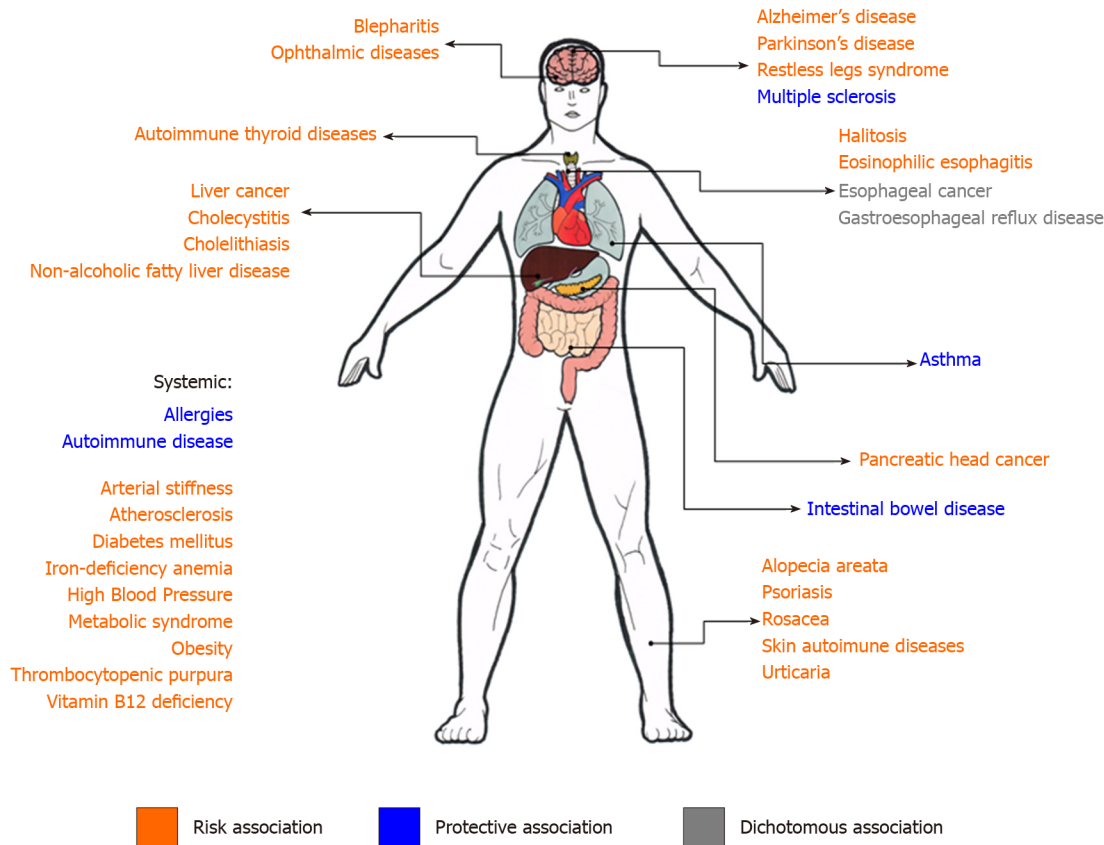


Figure 2 Summary scheme of non-gastric manifestations of *Helicobacter pylori* infection. In orange, the manifestations for which *Helicobacter pylori* (*H. pylori*) infection represents a risk association. In green, the manifestations for which *H. pylori* infection represents a protective association. In gray, the manifestations for which studies show a dichotomous association.

showed a rise in endoscopic reflux esophagitis incidence among treated patients (OR = 1.62, 95%CI: 1.20-2.19, $P = 0.002$). However, the occurrence of GERD symptoms was not significantly different between the groups (OR = 1.03, 95%CI: 0.87-1.21, $P = 0.76$), suggesting that the *H. pylori* eradication does not influence GERD symptoms onset. Regarding esophageal adenocarcinoma, which is usually due to GERD, a recent meta-analysis that included 35 studies showed that *H. pylori* infection may reduce the risk of development of that cancer (OR = 0.71, 95%CI: 0.57-0.92)^[27]. Meantime, these studies did not distinguish the patients infected by CagA-positive *H. pylori* from those colonized by CagA-negative bacteria.

Halitosis

Another non-gastric manifestation suggested to be related to *H. pylori* infection is halitosis^[28]. In 2017, HajiFattahi *et al*^[29] tried to prove this correlation, showing that among patients with halitosis, 91% were *H. pylori*-positive, against only 32% in the control group ($P < 0.001$). However, knowing that halitosis is associated with poor oral hygiene conditions, it is possible that there is a bias related to theory of hygiene in this association and, in this sense, studies have tried to prove this relationship and its pathophysiology, aiming biases exclusion^[30].

NAFLD

NAFLD refers to a range of disorders in which hepatic steatosis is observed by means of image or histology exams^[31]. It is believed that the above-mentioned condition is promoted by the insulin resistance induced by molecules whose production is stimulated by *H. pylori* infection such as tumor necrosis factor and C-reactive protein^[32]. Furthermore, a reduced production of adiponectin, a molecule that inhibits the fatty acid deposition in the liver, is observed in *H. pylori* patients^[33]. Moreover, the bacterium can reach the liver through the biliary tree and can lead to liver inflammation^[34,35]. Recently, studies have been developed in order to verify if *H. pylori* infection plays a role in that disease. Indeed, a meta-analysis that included 21 studies observed a positive association between this infection and NAFLD (OR = 1.529, 95%CI:

1.336-1.750, $P = 0.000$)^[36]. It is important to be highlighted that most available studies on this issue took place in Asian countries, so this data should be interpreted with caution when considered the Western population.

Hepatic carcinoma

Although researches have investigated the association between *H. pylori* infection and liver carcinoma, conflicting results have been found. However, it was already shown that *H. pylori* infection is associated with liver inflammation, fibrosis, and necrosis. Along with these repercussions, the bacterial translocation through the biliary tract may also lead to direct hepatic damage, predisposing or even triggering the carcinogenic process^[37,38]. The rates of *H. pylori* infection among HBV-related hepatic carcinoma patients (68.9%) and HBV-negative hepatic carcinoma (33.3%) were higher when compared to control groups ($P < 0.001$)^[39]. In this sense, studies agree with regards to the screening of *H. pylori* infection followed by the bacterial eradication in patients with liver disorders, in order to prevent the progression of the preexisting disease and the cancer onset^[39,40].

Cholecystitis and cholelithiasis

Recent research has investigated the possible risk relationship between *H. pylori* infections and the development of cholelithiasis and cholecystitis^[41,42]. Regarding the first one, studies have shown that the presence of *H. pylori* in bile may be a risk factor for its development^[43]. Moreover, among other studies, a meta-analysis demonstrated a positive association between *H. pylori* infection and chronic cholecystitis/cholelithiasis (OR = 3.022; 95%CI: 1.897-4.815; $P = 20.1\%$)^[44-46]. Among the possible explanations for that phenomenon, it is believed that *H. pylori* may infects the biliary system, causing chronic inflammation in its mucosa and, as a result, leading to the impairment of acid secretion and reduction of the dissolvability of calcium salts in bile, what predisposes the formation of gallstones^[44].

B12 deficiency

A probable risk relationship between *H. pylori* infection and pernicious anemia was also suggested^[47]. Case-control and prospective cohort studies have shown that patients with positive *H. pylori* had lower Vitamin B12 (Cobalamin) levels when compared to control groups^[47,48]. In addition, when treated with triple therapy - clarithromycin, amoxicillin and omeprazole - to eradicate *H. pylori*, patients with previous pernicious anemia obtained satisfactory levels of Vitamin B12, with mean iron levels of 262.5 ± 100.0 pg/mL among *H. pylori*-positives against 378.2 ± 160.6 pg/mL in the group of *H. pylori*-negatives, representing a difference of 30.6% between those groups, with a P value of 0.001^[48]. Corroborating to the consolidation of this association, studies have shown that there is a decrease in Cobalamin levels in *H. pylori* positive patients regardless of gastric atrophy and dyspepsia^[49]. Even though there is still a lack of studies with regard to clarifying the pathophysiological process of this risk association, the Maastricht V/Florence Consensus Report^[50] recommends that in patients with this deficiency, *H. pylori* should be sought and eradicated.

Iron deficiency anemia

Choe *et al.*^[51] conducted a randomized case-control study to test whether anemic patients positive for *H. pylori*, when underwent infection eradication therapy, have a better response of blood iron levels when compared to the control group. The results were positive for the risk association between infection and anemia. Since then, studies have tried to understand the pathophysiology behind this manifestation, in addition to evaluate its occurrence in different age groups.

In this scenario, subsequent studies confirmed this correlation, in addition to explaining that it occurs regardless of bleeding. That is, there is no need for tissue damage and hemorrhagic processes for the onset of anemia due to infection by *H. pylori*^[52]. When comparing *H. pylori* positive patients to the *H. pylori* negative ones, the first group had iron levels of 71.6 ± 24.8 µg/dL against 80.1 ± 20.7 µg/dL of the second one, a difference of 10.6%, with a t value of -3.206 and P value: 0.001^[48].

It has also been suggested that the anemia triggered by *H. pylori* infection is a causal factor for growth disorders among children and adolescents. Although this is a difficult relationship to be proven, some studies agree on the influence of anemia triggered by *H. pylori* as a causal factor for developmental gap among infants^[52,53]. In this sense, groups of children with unexplained anemia and growth disorders presenting clinical manifestations suggestive of infection by this bacterium should be screened and, if necessary, undergo *H. pylori* eradication, as recommended by current

guidelines^[50].

Dermatological and autoimmune diseases

Some studies suggest an association and possible causality of *H. pylori* infection in some dermatological diseases^[8]. Among them, rosacea and some immunological diseases such as idiopathic thrombocytopenic purpura, psoriasis, alopecia areata, and urticaria are the most studied ones. However, the evidence makes it clear that significant associative power with *H. pylori* infection only occurs in some of these diseases, whereas in many of them conflicting results have been obtained, demanding further research with more appropriate methodologies and statistical designs^[54]. As for autoimmune diseases, they are characterized by a dysregulation of the immune system, which leads to loss of tolerance to auto antigens^[55]. It is believed that these diseases have a multivariate etiology and that infectious agents can trigger them. The immunological response against *H. pylori* can generate an inflammatory condition that potentially leads to the development of cross-reactive antibodies^[56].

Rosacea is a chronic disease with skin manifestations such as facial erythema, edema, papules, telangiectasia, and pustules that are located, most of the time, in the center of the face^[57]. A risk association has been observed between *H. pylori* infection and rosacea, and the treatment of this bacterial infection dramatically decreases the severity of such dermatological disorder^[58]. Other authors observed the same associative results, and began to recommend that patients with rosacea who are positive for *H. pylori* should be treated with bacterial eradication^[59-61]. However, a meta-analysis concluded that cause-effect associations are weak between this disease and *H. pylori* infection (OR = 1.68, 95%CI: 1.100-2.84, $P = 0,052$) and that *H. pylori* eradication therapy does not reach the statistical significance necessary for its mass recommendation, (RR= 1.28, 95%CI: 0.98-1.67, $P = 0,069$)^[62]. The contrast of the results found in the literature may be related, among other things, to the big variability in methodological and statistical designs used.

Psoriasis is a chronic, non-contagious inflammatory skin disease, with genetic and autoimmune characteristics that affects the skin and joints^[63,64]. Its association with *H. pylori* infection had already been investigated with the search for antibodies against *H. pylori* in patients with psoriasis without known gastrointestinal complaints^[65]. Recently, a meta-analysis found a strong evidence demonstrating this association (OR = 1.19, 95%CI: 1.15-2.52, $P = 0.008$) and highlighted that the rate of *H. pylori* infection, interestingly, was significantly high in patients with moderate and severe psoriasis (OR = 2.27; 95%CI: 1.42-3.63, $P^2 = 27\%$) but not in patients with the milder disease (OR = 1.10; 95%CI: 0.79-1.54, $P^2 = 0\%$)^[66]. Another disease commonly associated with *H. pylori* infection is chronic urticaria, a clinical condition that presents with itchy, erythematous or swollen urticaria^[67,68]. The studies reveal conflicting evidence regarding the cause-effect association of *H. pylori* with chronic urticaria. Interestingly, a meta-analysis showed that the improvement in chronic urticaria was not directly linked to the eradication of *H. pylori*, but with the antibiotic therapy used, and, even if the treatment was not effective, a significant remission in chronic urticaria was observed in those patients^[69,70].

Alopecia areata (AA), an autoimmune disease, leads to hair loss and can present a variable course among affected individuals^[71]. There are few published studies on the association of AA with *H. pylori* infection. In an Iranian case control study, a statistically significant risk association was observed (OR = 2.263, 95%CI: 1.199-4.273); however, the study limitations such as the incapacity of controlling some confounding variables weaken this evidence^[72].

Idiopathic thrombocytopenic purpura (ITP) is a condition that results from the individual's platelet destruction mediated by antiplatelet antibodies^[73]. Several studies associate the relationship between *H. pylori* infection and ITP. Although the pathogenesis involved in this process is inconclusive, some authors suggest that CagA stimulates the synthesis of anti-CagA antibodies that cross-react with platelet surface antigens causing ITP^[74,75]. The first correlation of this possible pathogenesis observed an increase in patients' platelet count after *H. pylori* eradication^[76]. Other studies have also been conducted in order to evaluate the remission of PTI after the treatment of *H. pylori* infection. A prospective Brazilian study demonstrated an increase in platelets after bacterial eradication in part of the *H. pylori*-positive patients with ITP. In addition, a significant decrease in the levels of cytokines of the pro-inflammatory profiles Th1 and Th17 as well as an increase in anti-inflammatory cytokines linked to regulatory T cells (Treg) and Th2 were observed in infected patients with ITP in whom an increase in platelet count after *H. pylori* eradication was observed^[77]. A recently published meta-analysis corroborates the significant therapeutic effect that *H. pylori* eradication has on patients with ITP and suggests that this evidence can be taken into

account in the clinical treatment of patients with ITP (OR = 1.93, 95%CI: 1.01-3.71, $P = 0.05$)^[78]. This study presents fragilities since it included researches with a limited number of individuals, few studies with adults, and embraced a small variation of ethnicities. However, it is important to highlight that *H. pylori* infection investigation and eradication have been recommended by ITP clinical management guidelines^[79].

Ophthalmic manifestations

Ophthalmic manifestations association with *H. pylori* was firstly studied by Mindel and Rosenberg^[80], when they tried to relate the rosacea's ocular manifestations with this bacterium. The ocular and extraocular microbiomes and their influence in ophthalmic diseases have been extensively studied and although some associations with *H. pylori* infection are controversial, a set of diseases as open-angle glaucoma, central serous chorioretinopathy (CSCR), and blepharitis have been more widely studied^[81,82].

Cytokines induced by the *H. pylori* in gastric mucosa can generate a systemic inflammatory status contributing with the pathogenesis of these diseases through increased oxidative stress, causing mitochondrial dysfunction and damage to DNA. This process culminates in morphological changes and apoptosis. Oxidative stress is an important pillar in the pathogenesis of both conditions but it is a controversial causal association without large-scale studies^[82].

A meta-analysis showed a significant correlation between *H. pylori* infection and open-angle glaucoma (OR = 2.08, 95%CI: 1.42-3.04). Analyzing the subgroups, this association was present in primary open-angle glaucoma (OR = 3.06, 95%CI: 1.76-5.34; $P < 0.001$) and normal tension glaucoma (OR = 1.77, 95%CI: 1.27-2.46; $P = 0.001$), but not seen with pseudoexfoliation glaucoma (OR = 1.46, 95%CI: 0.40-5.30; $P = 0.562$)^[83]. The *H. pylori* eradication can result in an improvement of intraocular pressure ($P < 0.001$) and visual field ($P \leq 0.01$) parameters^[84]. The eradication had been either associated with the reduction of ocular rosacea symptoms in a case series^[85], improvement in patients with CSCR^[86] and better cytology results in 50% of patients with *H. pylori* and blepharitis ($n = 142$)^[87]. Regarding CSCR, studies have shown a higher prevalence of the disease among *H. pylori* positive patients (78.2%, 95%CI: 56% - 92%) when compared to the control group (43.5%, 95%CI: 23%-65%), with $P < 0.03$ and a 4.6 OR^[88].

Asthma and allergic diseases

The research on infections, microbiome and allergic diseases started with the discussion about hygiene hypothesis due to the increase of allergic diseases as allergic rhinitis or hay fever, asthma and eczema in the post industrial revolution world^[9]. The first study that aimed to determine the seroprevalence of *H. pylori* in asthma patients was performed in 2000 and had inconclusive findings^[89]. Therefore, several studies were conducted in an attempt to elucidate the association between both conditions and although some findings are controversial, meta-analyses indicate that *H. pylori* infection could be considered a protective factor for asthma especially in children and in patients with cagA-positive strains^[90,91].

The *H. pylori* infection as other microbial antigens tends to induce, especially in adults, a Th1 polarized response. This pro-Th1 balance inhibits the activation of a Th2 immune response, fundamental in the asthma and allergies pathophysiology whereas eosinophilic activation and IgE production are dependent of IL-4 and IL-5^[92]. The neutrophil-activating protein of *H. pylori* (*H. pylori*-NAP) can induce this polarization *in vivo* and *in vitro* and could be a target in the development of a treatment or a prevention strategy for asthma and others allergic diseases^[93].

In children, the *H. pylori* infection produces a predominant Treg pattern^[94] that either suppress the Th2-mediated allergic response. Furthermore, the *H. pylori* IgG titre in children was inversely correlated with asthma severity^[95]. This response triggered against bacterial antigens is strong and could suppress responses to autoantigens and allergens^[96]. Another mechanism that could explain the lower incidence of asthma in *H. pylori* carriers is that the presence of the bacteria, especially CagA+ strains, protects against gastroesophageal reflux disease (GERD), reducing GERD-related asthma and the exacerbations associated with this condition^[97].

Considering that *H. pylori* infection is associated with poor household hygiene, some authors and studies endorse that the infection should be considered a biomarker for precarious condition instead of a specific protective factor for allergic diseases^[98].

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease that affects the central

nervous system (CNS), causing a multifactorial immune dysregulation that involves genetic and environmental factors^[99]. For the first time, in 2007, it was reported a negative association between *H. pylori* infection and MS^[100]. In that occasion, a Japanese study included patients with opticospinal MS (OSMS), conventional MS (CMS), and healthy controls (HC). The results showed a considerably lower *H. pylori* seropositivity in CMS patients (22.6%, $P < 0.05$) when compared to HC (42.4%, $P = 0.0180$) and OSMS individuals (51.9%, $P = 0.0019$). After that, various studies investigated such association and two meta-analyses were conducted in order to evaluate the possible protective effect of *H. pylori* infection to MS. The first one included 1902 patients and demonstrated a significantly lower infection prevalence among SM patients when compared to controls with the same age range and sex (OR = 0.59, 95% CI: 0.37–0.94, $P = 0.03$)^[101]. The second one embraced 2806 individuals and also found a reduced general prevalence of *H. pylori* infection in MS patients (24.66% *vs* 31.84%, OR: 0.69, 95% CI: 0.57–0.83, $P < 0.0001$)^[102].

Among the various hypothesis that try to justify the *H. pylori* infection as a protection factor for MS, the hygiene hypothesis argues that the exposition to microbial agents during childhood modulates the human immune system, avoiding the development of immune hypersensitivity during adulthood^[9]. Another mechanism that is probably associated with this process is the inhibitory induction of *H. pylori* over the Th1 and Th17 immune response by means of the FoxP3-positive regulatory cells^[103].

Interestingly, the immune response against the *H. pylori* infection also seems to be influenced by the MS. In a cohort that included 119 MS patients (most of them with acute remittent-recurrent MS), it was demonstrated that the *H. pylori* positive patients presented a reduced humoral response against the bacterial protein HP986^[104]. However, another study investigated the antibodies production against the *H. pylori* VacA (vacuolating cytotoxin A) in patients with secondary progressive MS, who presented such immunoglobulins more often when compared to healthy individuals. This suggests that the recognition of *H. pylori* antigens by antibodies is influenced not only by the positivity status for EM, but also by the forms of presentation of this autoimmune disease^[105].

Alzheimer's disease

Alzheimer disease (AD) is a neurodegenerative disturb with progressive cognitive impairment and has the gradual involvement of the sporadic memory as its commonest clinical representation^[106]. Shindler-Itskovitch *et al*^[107], 2016, conducted a meta-analysis that embraced 13 observational studies on the association between *H. pylori* and dementia. The results showed that the *H. pylori*-positive patients had higher risk of dementia when compared to the not infected ones (OR = 1.7, 95% CI: 1.17–2.49). However, when considered only AD patients, such association was not statistically significant (OR = 1.39, 95% CI: 0.76–2.52). Despite that, a more recent meta-analysis identified a significant positive association between *H. pylori* infection and AD in an Asian population (OR = 1.60, 95% CI: 1.20–2.15)^[108].

Some mechanisms are supposed to be involved in the increase of AD risk in individuals infected by *H. pylori*. The vitamin B12 deficiency due to gastric alterations induced by the infection leads to increased concentrations of homocysteine, what leads to dementia. Other hypothesized mechanism for such association is the anormal hyperphosphorylation of the TAU protein caused by *H. pylori* infection. That protein is involved in the AD-linked neurodegeneration^[109]. Besides that, Kountouras *et al*^[110], showed that the *H. pylori* infection positively influence the ApoE polymorphism known as the mais genetic risk factor for AD.

Some hypotheses affirm that *H. pylori* can reach the brain, leading to changes that trigger AD. One of them is based on the *H. pylori* ability to reach the olfactory bulb through the oral-nasal-olfactive *via*^[111]. Such bulb is responsible for the decodification of olfactive signals in the brain and its dysfunction is related to the enfecalic neurodegeneration. Other supposition is the bacterial access through the rupted hematoencephalic barrier (HEB) inside leukocytes, causing an inflammatory process with the release of chemical mediator^[112]. All of the above-mentioned *H. pylori* ways to reach CNS could allow *H. pylori* to exert its potential neurodegenerative action in that environment^[113].

Parkinson's disease

Parkinson's disease (PD) is the second most prevalent progressive neurodegenerative disturb in the world, and has tremor, postural instability, and bradykinesia as preeminent outcomes^[114,115]. However, among other reverberations in the human body, gastrointestinal impairments, such as constipation, are important consequences of that

disease and they often represent the first PD manifestations^[116]. The supposed link between Parkinson's disease and gastrointestinal tract led researchers to further investigate that relationship, raising the hypothesis that microorganisms from the digestive system could influence PD pathophysiology^[117].

In that context, a recent meta-analysis that included 23 studies investigated the impact of viral, fungal, and bacterial infections in the risk of PD and found a positive association between *H. pylori* infection and that disease (pooled OR, 95%CI: 1.653, 1.426-1.915, $P < 0.001$)^[118]. Furthermore, another meta-analysis which included 7 studies found that the *H. pylori* infection is also associated with the clinical severity of the PD, since *H. pylori*-positive patients presented poorer scores when undergone Unified Parkinson Disease Rating Scale (UPDRS) evaluation [mean \pm SD, 95%CI: 6.83, 2.29-11.38, $P = 0.003$]^[119]. In addition, the last study also observed an improvement in the UPDRS-III scale in PD patients after *H. pylori* eradication (mean \pm SD, 95%CI: 6.83, 2.29-11.38, $P = 0.003$). It is important to be highlighted that these researches present some limitations. Firstly, the included studies used different diagnostic criteria of PD, and the methods performed for the detection of *H. pylori* infection also varied. The use of ELISA for *H. pylori* infection diagnosis in some of these studies may have overestimated the number of positive individuals, since that test is often reagent even when performed months following a possible *H. pylori* spontaneous eradication. Last but not least, *H. pylori* infection is closely related to various socioeconomic factors, including hygiene. Consequently, some of the associations between *H. pylori* and PD could have been correlational and not causal^[118,119].

It is known that the *H. pylori* infection increases the synthesis of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)^[118]. Such substance can cause dopamine depletion, as well as damage to the substantia nigra, what can lead to PD. Concomitantly, *H. pylori* infection has been found to reduce levodopa absorption, what potentially has the exacerbation of PD symptoms as a consequence^[118].

Guillain-Barré syndrome

The association between the Guillain-Barré syndrome and the *H. pylori* infection have been widely studied and, recently, a meta-analysis about this issue confirmed this interrelation, proceeding with the analysis of the anti-*H. pylori* antibody in serum and cerebrospinal liquid (CSL). When the first one was analyzed, the antibodies prevalence in the patients that presented GBS was significantly higher when compared to those without GBS (OR = 2.31, 95%CI: 1.30-4.11, $P = 0.004$). In the CSL analysis, there was also a strong positive association between GBS and anti-*H. pylori* IgG (OR = 42.45, 95%CI: 9.66-186.56, $P < 0.00001$)^[120].

Cardiovascular diseases

Atherosclerosis is a ischemic disease caused by a chronic inflammatory process in the arterial wall and that can lead to other circulatory system diseases^[121]. Yang *et al*^[122] showed, in an animal model, a positive association between *H. pylori* infection and atherosclerosis. They also observed that CagA potentially stimulates the foam production inside macrophages, what contributes to the magnification of the atherosclerotic plaque and arterial dysfunction. In addition, the *H. pylori*-infected gastric epithelial cells-derived exosomes (Hp-GES-EVs) are absorbed by the plaques and CagA is released inside them. Such event exacerbates the obstructive inflammatory process and lead to *in vitro* and *in vivo* lesions^[122]. A south korean study that evaluated the relationship between *H. pylori* and cardiovascular risk factors concluded that this bacterial infection has a atherogenic potential once it seems to influence the lipidic profile of the patient. The results pointed to higher levels of total cholesterol and low-density lipoprotein (LDL), as well as to decreased high-density lipoprotein (HDL) in *H. pylori*-positive individuals^[123]. In that context, another recent study complemented that hypothesis, showing that the *H. pylori* eradication intensely contributed to the improvement of the lipidic parameters in dyslipidemic individuals by means of an increase in HDL levels and a drop in LDL/HDL ratio, which is a parameter used in the evaluation of the atherosclerosis risk^[124].

Another meta-analysis explored the association between *H. pylori* and myocardial infarction (MI), and concluded that *H. pylori* implies a higher risk of MI (OR = 2.10, 95%CI: 1.75-2.53, $P = 0.06$)^[125]. Interestingly, a study identified that people with IL-1 polymorphisms present higher inflammatory activity and higher chances of suffering from ST-segment elevation MI (OR = 2.32, 95%CI: 1.23-4.37, $P = 0.009$)^[126].

A study conducted in a Chinese population identified a high prevalence of arterial hypertension in *H. pylori* seropositive patients (OR = 1.23; 95%CI: 1.04-1.46)^[127]. One of the mechanisms that can explain that association is the production of inflammatory cytokines such as TNF- α , interleukin-6 and c-reactive protein induced by the *H. pylori*

[128]. These cytokines lead to insulin resistance, contributing to the total peripheral vascular resistance and to the atherosclerotic process. Both phenomena are related to the hypertension^[129].

The coronary artery disease (CAD) is characterized by a heart blood flow reduction in the as a result of obstructions of the coronary arteries due to their narrowing due to an atherosclerotic and/or a thrombotic process^[130]. A meta-analysis showed that the *H. pylori* infection is significantly related to higher odds of CAD (OR = 1.11, 95%CI: 1.01-1.22, *P* = 0.24)^[131].

According Tamura *et al.*^[132], the probable hypothesis to explain the positive association between *H. pylori* and CAD may be linked to the atrophic gastritis caused by the bacterial chronic infection, leading to a decreased absorption of vitamin B12 and folic acid in the gastrointestinal tract. This absorptive deficiency causes an increase in the circulant levels of homocysteine, which potentially contribute to the CAD development. In addition, a study conducted by Kutluana and Kilciler^[133], 2019, demonstrated that the reduced absorption of above-mentioned nutrients due to atrophic gastritis and gastric intestinal metaplasia during *H. pylori* infection also lead to an increase in the arterial stiffness.

Diabetes mellitus

A positive association between *H. pylori* infection and diabetes mellitus (DM) was found in a meta-analysis of 39 studies that included more than 20 thousands patients (OR = 2.00, 95%CI: 1.82-2.20, *P* = 0.07)^[134]. Besides that, the *H. pylori* infection not only increase the risk of DM, but it also impairs the satisfactory control of glycemic levels in DM patients. A meta-analysis that included 35 studies observed that the glycated hemoglobin A levels were significantly higher in *H. pylori*-positive patients when compared to *H. pylori* negative individuals (weighted mean difference 0.50, 95%CI: 0.28-0.72, *P* < 0.001)^[135]. However, the fact that these studies do not take into account other factors that influence the glycemic control, such as obesity index and smoking status, constitute an important limitation^[134,135]. Among the hypothesis on how does *H. pylori* increases the risk of DM, it is believed that the increased cytokine production leads to the phosphorylation of serine residues from the insulin receptor substrate, whose linkage with insulin receptors turns deficient^[136].

CONCLUSION

Although *H. pylori* infection is most commonly associated with gastric manifestations, growing evidence have drawn attention to its role in extragastric diseases. The knowledge on how does that bacterium influence non-gastrointestinal disorders can elucidate little understood points about their pathophysiology and may shed light on new therapeutic targets in the management of these conditions. The *H. pylori* eradication is already a well established therapeutic alternative in some of these diseases. However, further studies are needed in order to evaluate if the bacterial elimination can be a consistent therapeutic alternative in a greater number of health problems. Finally, the beneficial association of *H. pylori* infection with some extragastric diseases should be explored by future research in order to evaluate the use of the bacterium and its products in new prophylactic and therapeutic protocols.

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Basic Study

Celecoxib attenuates hepatocyte apoptosis by inhibiting endoplasmic reticulum stress in thioacetamide-induced cirrhotic rats

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Abstract

BACKGROUND

Endoplasmic reticulum (ER) stress is an important mechanism in the progression of chronic and acute liver diseases, especially in the progression and recovery of liver fibrosis. Excessive and long-term ER stress induces apoptosis. ER stress-induced apoptosis is considered to be an important pathway in the development of liver fibrosis. Cyclooxygenase-2 (COX-2) induction is also closely related to ER stress. In our previous studies, we showed that celecoxib, a COX-2 inhibitor, improves liver fibrosis and portal hypertension. However, the role and mechanism of celecoxib in alleviating liver fibrosis remain unclear.

AIM

To investigate whether celecoxib alleviates liver fibrosis by inhibiting hepatocyte apoptosis *via* the ER stress response.

METHODS

Cirrhosis was induced by intraperitoneal injections of thioacetamide (TAA) for 16 wk (injection dose is 200 mg/kg per 3 d for the first 8 wk and 100 mg/kg per 3 d after 8 wk). Thirty-six male Sprague-Dawley rats were randomly divided into three groups, namely, control group, TAA group, and TAA + celecoxib group. In the last 8 wk, TAA-induced cirrhotic rats received celecoxib (20 mg/kg/day) or the vehicle by gastric gavage. After 16 wk, the rats were sacrificed, and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin

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(ALB) were detected. The hepatic fibrosis areas were evaluated by Sirius red staining and the degree of fibrosis was assessed by measuring the level of hydroxyproline. ER stress levels were evaluated by detecting the marker proteins glucose-regulated protein 78 (GRP78), CCAAT/enhancer binding protein homologous protein (CHOP), PKR-like ER protein kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 alpha (IRE1α). Apoptosis levels were evaluated by detecting caspase-12 and caspase-3.

RESULTS

The serum ALT and AST levels in the liver were significantly reduced by celecoxib; however, the serum ALB had no significant changes. Celecoxib significantly reduced the degree of liver fibrosis and the levels of hydroxyproline (-38% and -25.7%, respectively, $P < 0.01$). Celecoxib ameliorated ER stress by reducing the level of GRP78 compared to the TAA group ($P < 0.05$). Consistently, after celecoxib administration, the upregulation of TAA-induced hepatic apoptosis markers (caspase-12 and caspase-3) and CHOP were significantly inhibited. In addition, after celecoxib treatment, the expression of key molecules associated with ER stress (PERK, ATF6, and IRE1) was decreased ($P < 0.05$).

CONCLUSION

Therapeutic administration of celecoxib effectively reduces hepatic apoptosis in TAA-induced cirrhotic rats. The mechanism of action may be attributed to the suppression of CHOP expression, which subsequently inhibits ER stress.

Key words: Liver fibrosis; Endoplasmic reticulum stress; Celecoxib; Cyclooxygenase-2; CCAAT/enhancer binding protein homologous protein; Apoptosis

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Core tip: This study aimed to explore the important role of celecoxib in modulating hepatocyte apoptosis during the development of liver fibrosis, and to clarify that the mechanism of its regulation is mediated by endoplasmic reticulum stress, which in turn affects the progression of liver fibrosis. We concluded that therapeutic administration of celecoxib can effectively reduce hepatic apoptosis in thioacetamide-induced cirrhotic rats. The mechanism of action may be attributed to the suppression of CCAAT/enhancer binding protein homologous protein expression, which subsequently inhibits endoplasmic reticulum stress.

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INTRODUCTION

Liver fibrosis is the outcome of hepatocellular injury-repair reactions caused by chronic viral hepatitis, alcoholism, autoimmune diseases, genetic and metabolic disorders, and repeated and sustained liver injury^[1,2]. The pathological process of liver fibrosis is based on long-term, sustained action of various injuries, including hepatic parenchymal cell necrosis, apoptosis, and collagen synthesis, which leads to abnormal deposition of the extracellular matrix (ECM) in liver tissue, a pathological process that further triggers abnormal changes in liver structure and liver function. Hepatic stellate cells (HSCs) play a significant role in maintaining liver homeostasis, and they will undergo phenotypic conversion into myofibroblast cells under sustained liver injury, which express smooth muscle actin, cause ECM deposition, and promote liver fibrosis^[3,4]. Additionally, the presence and exacerbation of inflammation are another important factor in the occurrence and development of liver fibrosis^[5,6]. It is known that liver fibrosis can be reversed after removing the damage factors. Therefore, to explore a novel and targeted therapy is very useful for the treatment of liver fibrosis.

Recently, many studies have shown that hepatocyte apoptosis is the most basic central link that causes liver damage and liver disease^[7]. There is plenty of endoplasmic reticulum (ER) in liver cells, and many liver diseases are related to ER stress and ER stress-mediated apoptosis, such as viral hepatitis, liver fibrosis, non-alcoholic fatty liver disease, alcoholic liver disease, acute liver failure, drug-induced liver disease, and liver cancer. A variety of metabolic, toxic, and inflammatory injuries can cause liver damage and disease. A common feature of these injuries is the activation of apoptosis and/or necrosis^[8,9].

Therefore, inhibiting hepatocyte apoptosis and reducing liver tissue damage and the severity of inflammation are of great importance in delaying the progression of liver fibrosis. Recent studies have shown that ER stress is a critical event in the development of liver fibrosis^[1,2,5,8]. However, the functional alteration in ER stress and its potential role in liver fibrosis are still unclear. The ER stress response is a self-protective mechanism for cell adaptation, but excessive or long-lasting ER stress causes apoptosis^[10].

The ER has a high dynamic balance under normal circumstances. However, ER stress is triggered in response to various physiological or pathological stimuli, especially when the ability of accumulation of unfolded proteins within the ER exceeds the folding capacity of ER chaperones^[11,12]. ER stress activates the adaptive unfolded protein response (UPR) and promotes the molecular chaperone protein glucose-regulated protein 78 (GRP78) dissociation^[13]. ER stress or UPR transducers contain three transmembrane proteins: PKR-like ER protein kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 alpha (IRE1α). When the ER stress response is started, the signaling proteins PERK, ATF6, and IRE1α are activated and dissociate from GRP78, thereby activating the UPR. Apoptosis occurs by increasing the activity of the proapoptotic transcription factor C/EBP homologous protein [CCAAT/enhancer binding protein homologous transcription factors (CHOP)] under prolonged ER stress, which rapidly increases through activation of the above-mentioned signaling proteins. Various injury factors activate the ER stress response, leading to liver fibrosis, atherosclerosis, ischemic heart disease, and myocardial injury^[12,14,15]. Therefore, the search for drugs that effectively inhibit the ER stress response is of great significance for the treatment of liver diseases.

Extensive studies have confirmed that increased expression of cyclooxygenase-2 (COX-2) is related to the progression of fibrosis^[16]. Therefore, we hypothesized that COX-2 plays an important role in the formation of liver fibrosis, and selective COX-2 inhibitors may have antifibrotic effects^[17]. Our previous studies have shown that inhibition of COX-2 alleviates liver fibrosis and portal hypertension^[18]. However, the mechanism by which inhibition of COX-2 alleviates liver fibrosis is still unclear. Celecoxib is a common selective COX-2 inhibitor, and due to its safety and effectiveness, it is currently widely used in the treatment of rheumatism and osteoarthritis^[7]. Moreover, celecoxib has a pro-apoptotic effect on HSCs, the mechanism of which is to inactivate Akt in response to bile duct ligation and thioacetamide (TAA)^[19,20]. Our previous study showed that long-term use of celecoxib effectively improves portal hypertension in a rat model of TAA-induced cirrhosis because it has dual inhibitory effects on intrahepatic angiogenesis and fibrosis^[18]. In the present study, we explored the effects of celecoxib in reducing liver fibrosis by inhibiting the expression of biomarkers in the ER stress signaling pathway and reducing hepatic apoptosis in a rat model of TAA-induced cirrhosis.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were provided by the Experimental Animal Center of Sichuan University, which were reared under conditions of constant temperature and humidity with a 12-h light-dark cycle. Animal experiments were approved by the Animal Use and Nursing Committee of Sichuan University and conducted according to regulations formulated by Sichuan University.

Treatments

TAA (Sigma Chemical Company, St. Louis, Missouri, United States) was used to induce cirrhosis. The dose was 200 mg/kg per 3 d for 16 wk by intraperitoneal injection (i.p). Thirty-six male Sprague-Dawley rats weighing 180-230 g were randomly divided into three groups: Control, TAA, and TAA + celecoxib groups (12 rats per group). The administration of TAA + celecoxib (20 mg/kg per day, Pfizer,

New York, NY, United States) began with TAA administration. The TAA group was injected with TAA + placebo, and the control group was injected with normal saline (1 mL, i.p., every 3 d). One week after the last treatment, all rats were anesthetized to avoid acute toxic effects. After the rats were sacrificed, the liver was cut into two parts. One part was fixed in 4% neutral buffered paraformaldehyde for histopathological and immunohistochemical analysis, and the other part was frozen at -80°C for further analysis. Serum samples were also collected and frozen at -80 °C for further analysis.

Morphological study

The general morphology of the liver at the time of sacrifice was observed, and the degree of cirrhosis was evaluated. The liver tissue was embedded in paraffin, sectioned (3 µm thick), and stained with hematoxylin-eosin (HE) for histological evaluation and with Sirius red for assessment of collagen deposition. The Ishak scoring system was used to randomly evaluate the fibrosis degree in three randomly selected fields (100 × magnification) per section. Liver tissue for transmission electron microscopy (TEM; H-600IV, Hitachi, Tokyo, Japan) was processed according to standard methods for observing morphological changes.

Immunohistochemical study

Paraffin sections were incubated with 3% H₂O₂ at 37°C for 10 min. After blocking for 20 min at room temperature, the sections were incubated overnight with primary antibody at 4°C, followed by incubation with a secondary antibody for 30 min at 37°C. Finally, a signal detection system (ZSGB-BIO) with diaminobenzidine was used as the substrate for coloration, with brown staining indicating positive staining. Five fields were randomly selected from each group, with six rats in each group. Antibodies to GRP78, CHOP, ATF6, ATF4, PERK, IRE1, and X-box binding protein-1 (XBP1) were purchased from Santa Cruz Biotechnology (CA, US) and Proteintech Group, Inc. (Wuhan, China). The secondary antibodies were purchased from Santa Cruz Biotechnology.

Serum biochemistry for liver and renal function parameters

The Olympus AU2700 analyzer (Olympus, Hamburg, Germany) was used to detect serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, creatinine, and bilirubin levels according to the recommendations of the International Federation of Clinical Chemistry.

Hydroxyproline detection

Hydroxyproline is the main component of collagen, and the content of which is an important indicator of the degree of collagen metabolism and fibrosis. In this study, the modified classical acid decomposition method was used to calculate the hydroxyproline content, and the specific steps were carried out according to the kit instructions.

Western blot analysis of liver protein expression

The frozen liver tissue was homogenized in ice-cold RIPA buffer (Biosharp, Guangzhou, China) to prepare total protein extracts. Equal amounts (50 µg) of protein in each container were separated by 10% or 12% SDS-PAGE, and then transferred to PVDF membranes (Millipore, Billerica, MA, United States), followed by incubation with 2.5% skimmed milk powder in TBST Block (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl and 0.1% Tween-20). Using glyceraldehyde 3-phosphate dehydrogenase as an internal reference, the expression of immunoreactive protein was detected using an ECL detection kit (Beyotime).

Confirmation at the cellular level

The hepatocyte cell line L02 was cultured, and the COX-2 plasmid and vacuolar plasmid (control) were added after the cells were attached to the wall. After 24 h of transfection, tunicamycin (Tu) and thapsigargin (TG) were separately added to induce the occurrence of ER stress, followed by determination of ER stress channel-associated proteins (GRP78, XBP1, ATF4, ATF6, PERK, and IRE1), thereby inferring the relationship between COX-2 and ER stress. Meanwhile, the expression of ER stress pathway-associated proteins was detected as well after intervention with celecoxib.

Statistical analysis

All results are expressed as the mean ± SD and analyzed with SPSS 20.0 software (SPSS, Chicago, IL, United States). One-way ANOVA was applied for comparisons

among three or more groups. The Student-Newman-Keuls test was used to compare the difference between two groups. A P value < 0.05 was considered significant.

RESULTS

Celecoxib reduces hepatocyte damage and inhibits liver fibrosis

The effect of TAA on liver damage was evaluated by measuring the serum levels of AST and ALT. As shown in **Figure 1A**, the serum concentrations of AST and ALT in the TAA group were significantly increased compared with those in the control group, while the levels of both AST and ALT in the TAA group were higher than those in the TAA + celecoxib group, indicating that celecoxib significantly attenuated TAA-induced hepatocyte injury. In addition, the increased hydroxyproline concentration in the TAA group was significantly decreased in the TAA + celecoxib group. Severe pathological changes, such as structural rearrangement of hepatic lobules and formation of bridging fibrosis around cells, were shown by HE staining in the liver tissue of the TAA group, while these changes were significantly reduced in the TAA + celecoxib group. This result was also evidenced by damage to or the death of hepatocytes (**Figure 1**).

Liver sections of both the TAA group and TAA + celecoxib group showed a significant increase in collagen around the extracellular space, especially in the portal vein^[18]. However, compared with the TAA group, the collagen concentration in the TAA + celecoxib group was significantly reduced (**Figure 1B**).

Celecoxib reduces liver fibrosis and cirrhosis

Compared with the control group, a large amount of ECM accumulated in the liver of animals in the TAA group, causing structural disruption and destruction, and hepatocytes were lost, forming continuous fibrous septa, central venous distortion, and regenerative nodules, although celecoxib treatment reduced the progression of liver fibrosis significantly. The Ishak score and percentage of fibrotic area in the TAA group were significantly higher than those of the TAA + celecoxib group ($P < 0.05$ and $P < 0.01$). The liver of animals in the normal control group was soft and red in color, while the liver of animals in the TAA group showed cirrhosis that was characterized by extensive nodular and continuous fibrotic septa. These cirrhotic lesions were significantly relieved after treatment with celecoxib, resulting in a nearly normal liver appearance in the TAA + celecoxib group.

Celecoxib inhibits hepatocyte apoptosis by inhibiting caspase-12 and caspase-3 expression

As shown in **Figure 2A** and **B**, compared with the control group, the protein level of COX-2 was significantly increased in the TAA group, while COX-2 expression was significantly reduced after celecoxib administration. In addition, the collagen III protein level was examined by immunohistochemical staining and Western blot analysis, which showed that the collagen III-positive area was significantly reduced by celecoxib treatment. After TAA treatment, the expression of caspase-12 and caspase-3 increased in liver tissue, while it decreased significantly in the TAA + celecoxib group. These results indicated that celecoxib inhibits hepatocyte apoptosis by inhibiting the expression of caspase-12 and caspase-3 in fibrotic liver tissue and further suppresses the progression of liver fibrosis.

TEM analysis of liver sections showed severe damage to the mitochondria and ER in the TAA group, and the ER was swollen. In addition, broken ER and turbulent internal structures were observed in damaged hepatocytes (**Figure 3A**). After treatment with celecoxib, the mitochondria and ER of hepatocytes were protected, and the structure and morphology of the ER in hepatocytes were intact (**Figure 3A**). In addition, the marker proteins of ER stress were further examined by Western blot analysis, which showed that the expression levels of GRP78 and CHOP in the TAA group were obviously increased (**Figure 3B**), while their expression levels were obviously decreased after treatment with celecoxib. Collectively, these findings suggest that celecoxib inhibits ER stress in hepatocytes and further inhibits liver fibrosis by inhibiting the expression of GRP78 and CHOP.

Celecoxib attenuates hepatocyte apoptosis by inhibiting the expression of UPR-related pathway proteins

ER stress seems to exert its effects by activating the UPR signaling pathway. Therefore,

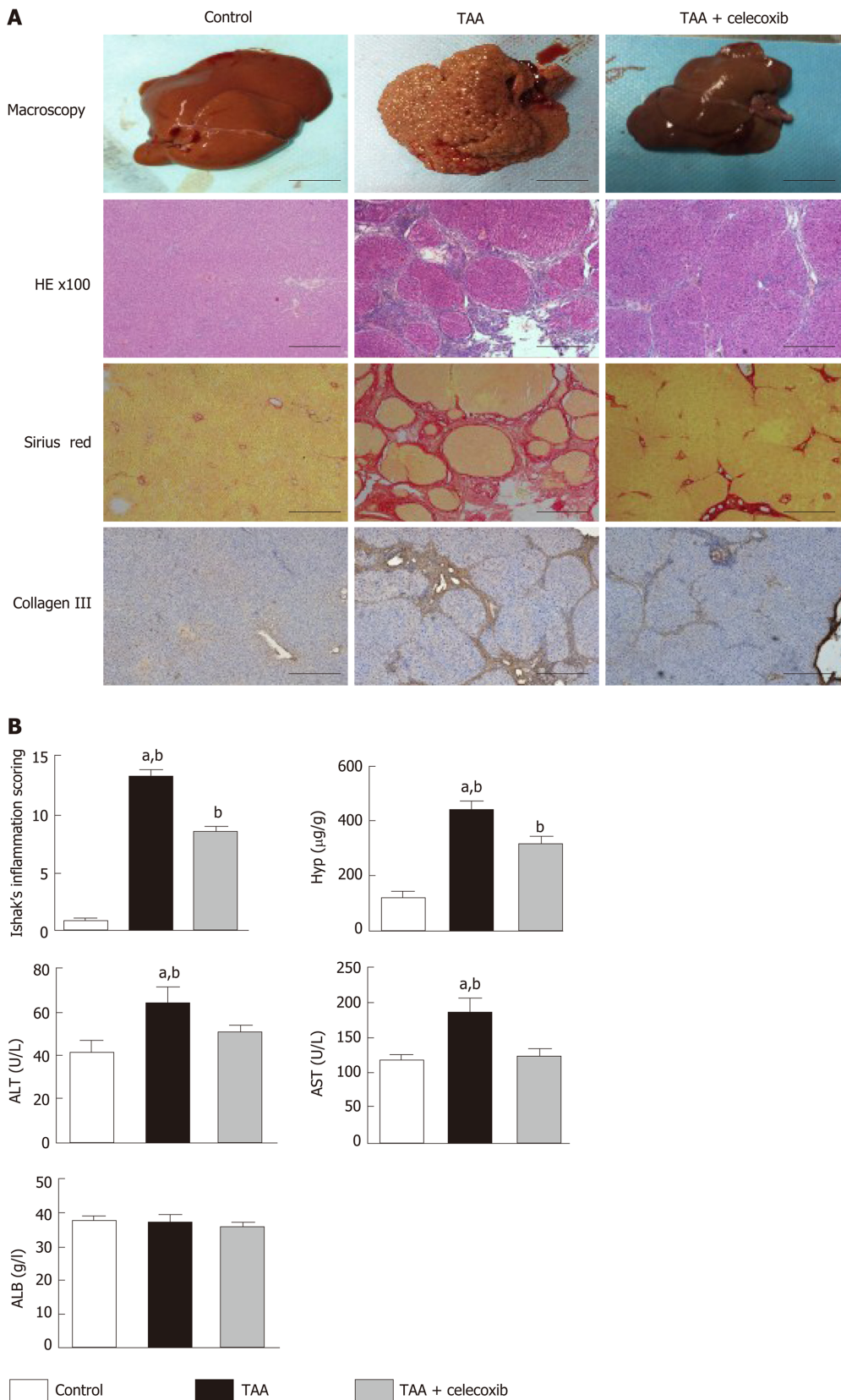


Figure 1 Effect of thioacetamide and celecoxib on liver fibrosis. A: Macroscopic analysis of liver tissue. Celecoxib improved pathological changes in the liver, as shown by hematoxylin-eosin and Sirius red (SR) staining (original magnification: $\times 100$; scale bar = 400 μm); B: Ishak's score based on histology and SR staining as well as alanine aminotransferase, aspartate aminotransferase, and hydroxyproline levels. The data are expressed as the mean \pm SD ($n = 15$, ^a $P < 0.05$ vs TAA + celecoxib group; ^b $P < 0.01$ vs control group). TAA: Thioacetamide; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HE: Hematoxylin-eosin staining.

the expression of UPR-related pathway proteins PERK, XBP1, ATF4, IRE1 and ATF6 was detected. The results showed that the expression levels of PERK, XBP1, ATF4,

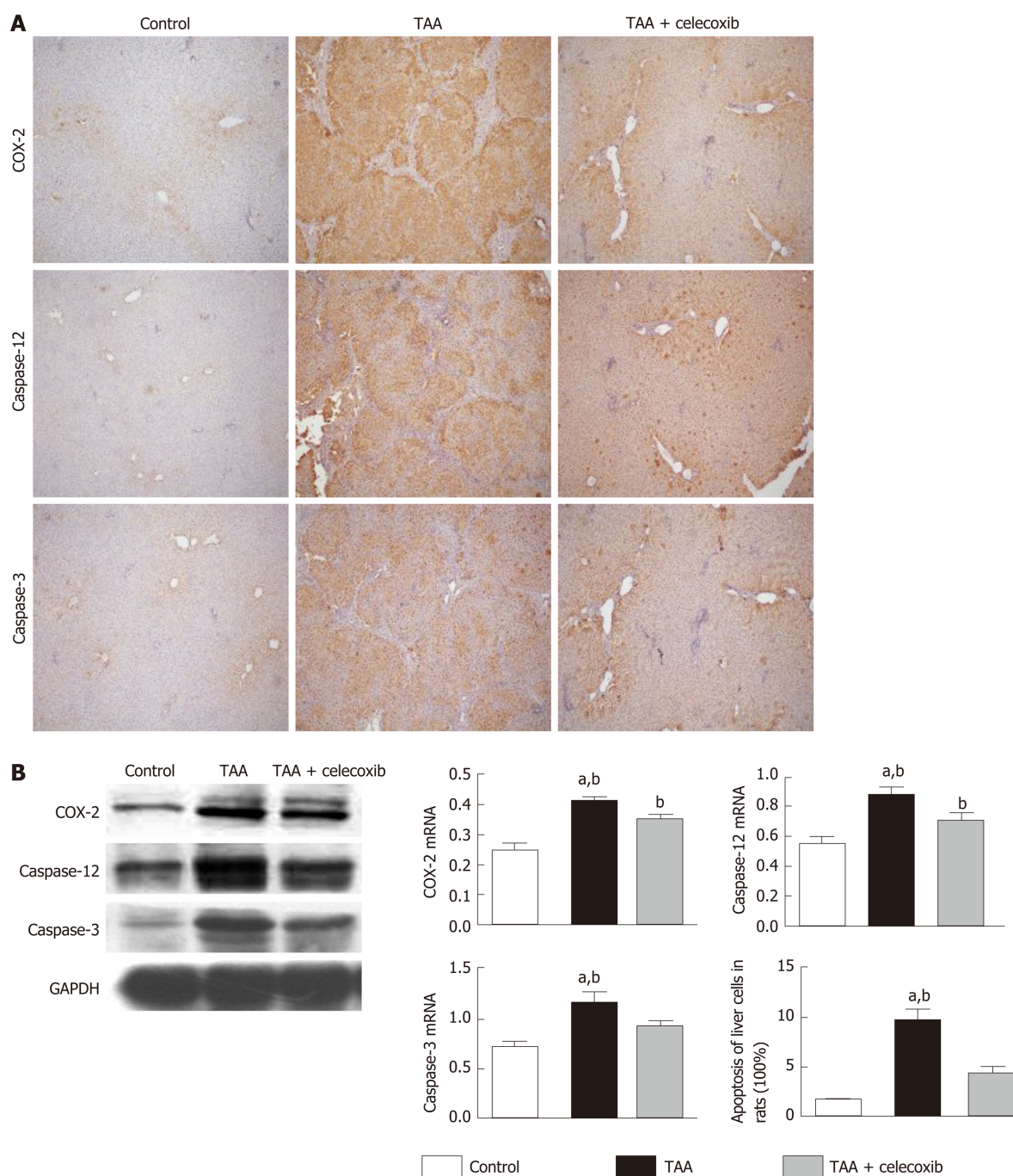


Figure 2 Celecoxib inhibits apoptosis of hepatocytes and expression of caspase-3 and caspase-12 in thioacetamide-induced liver fibrosis.

A: Immunohistochemical staining (100 ×) for collagen III, cyclooxygenase-2 (COX-2), caspase-12, and caspase-3 in the three groups of rats. Positive rates were analyzed using Image-Pro Plus 6.0. The data are expressed as the mean ± SD ($n = 15$, ^a $P < 0.05$ vs TAA + celecoxib group; ^b $P < 0.01$ vs control group). The scale bar is 200 μm; **B:** Western blot results showing collagen III, COX-2, caspase-3, and caspase-12 expression in the three groups. ^a $P < 0.05$ vs TAA + celecoxib group; ^b $P < 0.01$ vs control group. TAA: Thioacetamide; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

IRE1, and ATF6 were significantly increased in the TAA group than in the control group (Figure 4). After celecoxib treatment, the expression levels of these proteins were significantly reduced (Figure 4), suggesting that celecoxib inhibits hepatocyte apoptosis by inhibiting the expression of UPR-related pathway proteins in liver tissue.

Celecoxib inhibits ER stress in normal hepatocytes (L02) by regulating the expression of biomarkers in the ER stress signaling pathway

Liver fibrosis is a chronic inflammatory process with significantly increased expression of COX-2. Celecoxib, a selective COX-2 inhibitor, significantly attenuates liver fibrosis. However, it is worth exploring whether celecoxib plays a therapeutic role by

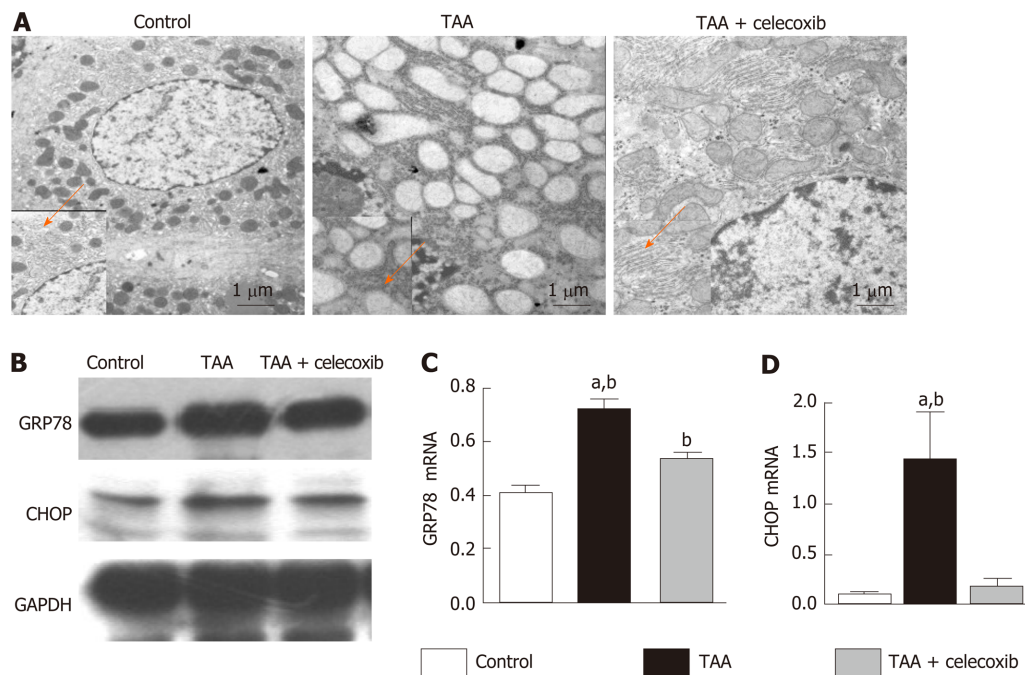


Figure 3 Celecoxib inhibits endoplasmic reticulum stress. A: Hepatocytes in the thioacetamide (TAA) and TAA + celecoxib groups (transmission electron microscopy, $\times 6000$ - 10000); B: Hepatic glucose-regulated protein 78 and CCAAT/enhancer binding protein homologous protein expression measured by Western blot. $n = 6/\text{group}$; C and D: Quantitative Western blot results. ^a $P < 0.05$ vs TAA + celecoxib group; ^b $P < 0.01$ vs the control group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GRP78: Glucose-regulated protein 78; CHOP: CCAAT/enhancer binding protein homologous protein. TAA: Thioacetamide.

influencing ER stress-related signaling pathways at the cellular level. To verify the correlation between COX-2 and ER stress, we transfected a COX-2 expressing plasmid into a cell model and added the ER stress inducers chlamycin and carotene to induce ER stress. The results showed that the ER stress level and the expression of proteins of related signaling pathways were obviously increased in the COX-2-pIRES2 group compared with those of the DMSO group and Empty-pIRES2 group (Figure 5A). After celecoxib treatment, the expression of GRP78 was significantly decreased in the Tu + celecoxib and TG + celecoxib groups (Figure 5B). In addition, celecoxib significantly decreased the expression of ATF6, PERK, and IRE1, but did not affect the expression of ATF4 in the Tu + celecoxib and TG + celecoxib groups, which strongly suggested that celecoxib may have a significant effect on the expression of biomarkers in the ER stress signaling pathway.

DISCUSSION

Several potential therapies have been proposed in the study of the mechanism of fibrogenesis using *in vitro* and *in vivo* models^[13,21,22]. Although treatments for underlying disease processes have been shown to be effective in reversing or reducing fibrosis, such as viral infections; however, no effective antifibrotic drugs have been found for humans^[23].

Therefore, it is essential to update our understanding of the mechanism of fibrosis and translate these findings to the development of new treatment options. First, animal models, especially rodent models, are still used to determine the target correlation, and an antifibrotic drug with a curative effect is an important tool^[24]. The animal models of liver fibrosis induced by chemicals such as carbon tetrachloride have been popular in previous studies^[25]. However, that model has serious hepatocyte necrosis and is dependent on a large amount of oxidative stress, which has not been found to be so severe in human chronic liver disease. Another chemical, TAA, is a hepatotoxin that induces apoptosis of hepatocytes. It has been shown to be helpful in detecting the protective effects of drugs, and the pattern of fibrosis is very similar to that of substantial fibrosis in humans^[26]. After 16 wk of intraperitoneal injection of TAA, the liver tissue of SD rats showed obvious inflammatory cell infiltration, disordered hepatocyte structure, hepatocyte degeneration and necrosis, and large

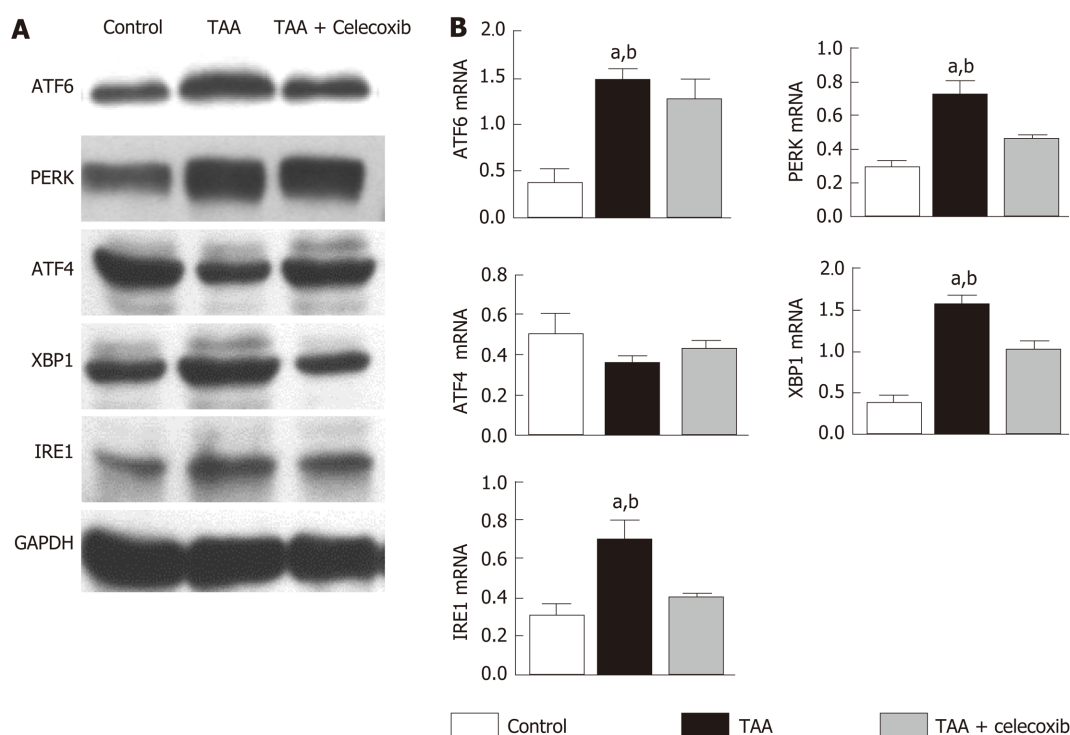


Figure 4 Celecoxib attenuates hepatocyte apoptosis by inhibiting the expression of unfolded protein response-related pathway proteins.

A: Hepatic activating transcription factor 6 (ATF6), PKR-like endoplasmic reticulum protein kinase, ATF4, X-box binding protein-1, and inositol-requiring enzyme 1 protein expression measured by Western blot. $n = 6/\text{group}$; B: Quantitative Western blot results. ^a $P < 0.05$ vs TAA + celecoxib group; ^b $P < 0.01$ vs control group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ATF6: Activating transcription factor 6; PERK: PKR-like endoplasmic reticulum protein kinase; XBP1: X-box binding protein-1; IRE1: Inositol-requiring enzyme 1; TAA: Thioacetamide.

amounts of collagen fiber deposition. The degree of liver fibrosis increased significantly, which indicated that the model of liver fibrosis was successfully established in this study. In addition, the expression levels of caspase-3 and caspase-12 in this liver fibrosis model increased significantly, suggesting that hepatocyte apoptosis occurs during liver fibrosis^[27].

The hepatocyte ER is the main place for cells to process proteins and store Ca^{2+} and closely related to substance transport, exchange, and detoxification^[28]. Protein misfolding and the accumulation of unfolded proteins in the lumen as well as disorders in Ca^{2+} balance have been observed when ER stress occurs^[29]. Several studies have demonstrated that ER stress is related to the progression of liver fibrosis and its recovery^[30]. Although GRP78, XBP1, and CHOP are not unique, these are all known indicators of the activation of the ER stress pathways^[31]. We revealed that the ER stress marker proteins GRP78 and CHOP increased significantly, indicating that an ER stress reaction occurred in the liver tissue of rats with liver fibrosis. ER stress exerts its physiological function mainly by activating the UPR signaling pathway. Our study also revealed that the expression of UPR signaling proteins in liver tissue of rats with liver fibrosis was significantly increased, which was consistent with the results reported by Volmer *et al*^[32].

The expression of COX-2 is significantly increased in the fibrotic liver tissues of animals and humans, and its expression level is positively correlated with the degree of fibrosis^[7]. Our previous studies confirmed that celecoxib can effectively alleviate liver fibrosis, but whether its mechanism is related to mediating the ER stress response has not been reported. Liver fibrosis is a chronic liver injury that is characterized by abnormal deposition of the ECM, which can progress to cirrhosis or even liver cancer^[33]. Therefore, it is of great significance to find drugs that can effectively alleviate liver damage. Celecoxib is a highly selective COX-2 inhibitor that has been shown to have antiproliferative effects in synovial fibroblasts as well as in human tumor xenograft models of breast, colon, lung, and prostate cancers and hepatocellular carcinoma^[34]. However, the principal mechanism by which celecoxib affects hepatocyte proliferation remains unclear. The results of this study indicated that the levels of ALT, AST, albumin, and hydroxyproline, the proportion of fibrotic liver tissue, Ishak score, and the expression levels of caspase-3 and caspase-12 were significantly

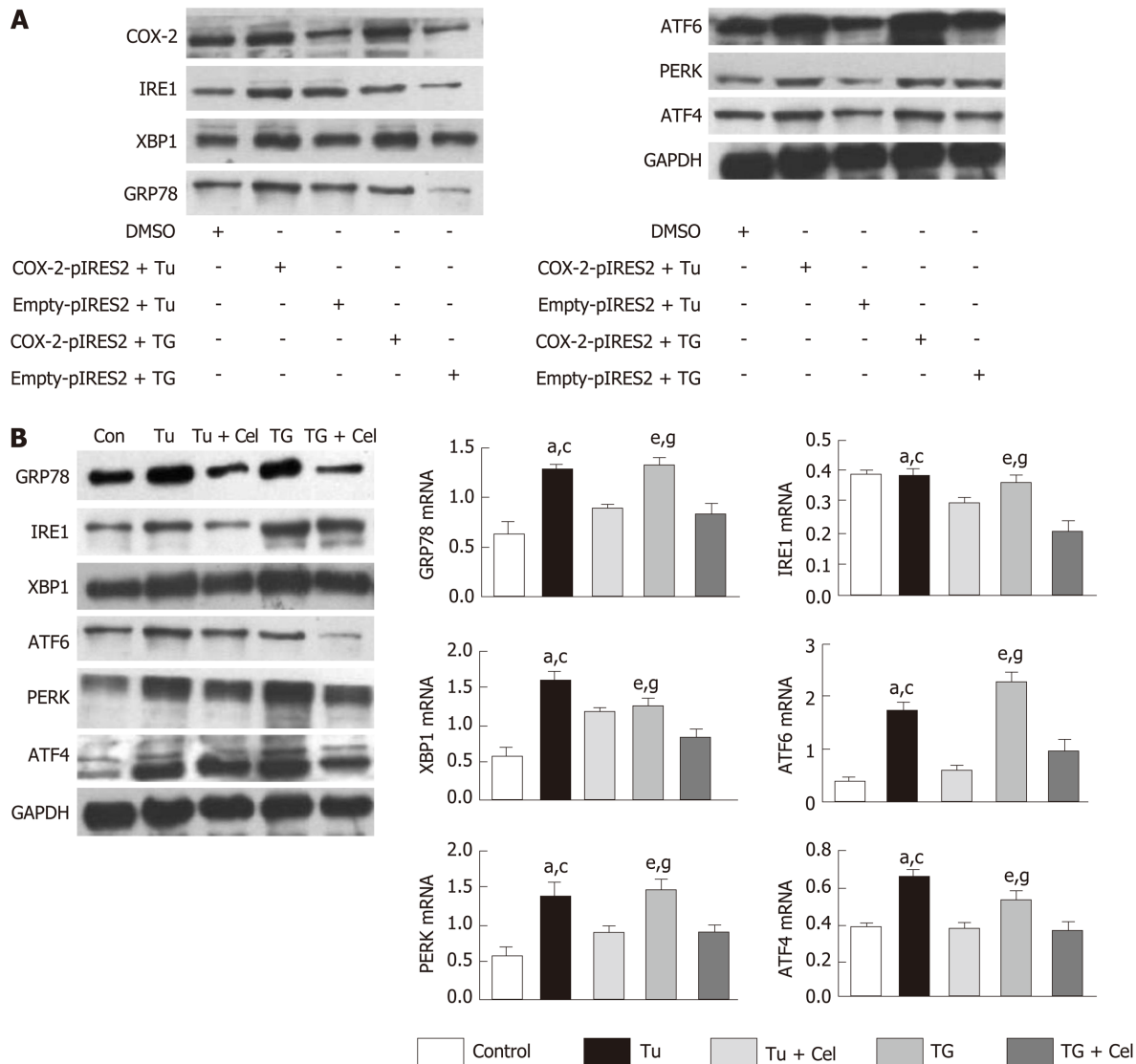


Figure 5 Celecoxib regulates the endoplasmic reticulum stress signaling pathway. A: Western blot analysis of glucose-regulated protein 78 (GRP78), inositol enzyme 1 (IRE1), X-box binding protein 1 (XBP1), activated transcription factor 6 (ATF6), PKR-like endoplasmic reticulum protein kinase (PERK), and ATF4 levels in L02 cells transfected with a cyclooxygenase 2 (COX-2) expressing plasmid and treated with endoplasmic reticulum stress inducers tunicamycin (Tu) and thapsigargin (TG); B: Western blot analysis of GRP78, IRE1, XBP1, ATF6, PERK and ATF4 levels in L02 cells treated with celecoxib and/or Tu and TG. ^a*P* < 0.05 between Tu group and control group, ^c*P* < 0.05 between Tu group and Tu + celecoxib group, ^e*P* < 0.05 between TG group and control group, ^g*P* < 0.05 between TG group and control group TG + celecoxib group (*n* = 6). Con: Control; Tu: Tunicamycin; TG: Thapsigargin; Tu + Cel: Tu + celecoxib; TG + Cel: TG + celecoxib; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GRP78: Glucose-regulated protein 78; ATF6: Activating transcription factor 6; PERK: PKR-like endoplasmic reticulum protein kinase; XBP1: X-box binding protein-1; IRE1: Inositol-requiring enzyme 1; TAA: Thioacetamide; COX-2: Cyclooxygenase-2.

decreased after celecoxib intervention, which strongly suggested that celecoxib attenuates liver fibrosis and inhibits hepatocyte apoptosis, thereby protecting hepatocytes.

CHOP is considered the major determinant of cell fate and ER stress-induced apoptosis^[35]. It is a short-lived protein, and so under mild or transient ER stress, the expression of CHOP will not change, and cells adapt and recover^[36]. But if the ER stress intensity is strong or duration is long, persistent CHOP expression and cell death will occur^[37]. In the present study, we revealed for the first time that the expression of key ER stress molecules, such as CHOP, was obviously decreased after celecoxib treatment, indicating that celecoxib plays a role in inhibiting ER stress in rat liver fibrosis by inhibiting the expression of CHOP and the ER stress signaling pathway.

Liver fibrosis is an important sign of chronic liver diseases, which could be treated and even reversed at early stage^[19]. At present, the gold standard method for diagnosing liver fibrosis is liver biopsy^[38]. However, sampling error and potential complications are the limitations of this method. In recent years, non-invasive

diagnostic techniques and molecular imaging quantitative detection technology have developed rapidly, and their application in quantitative detection of liver fibrosis has also received more and more attention^[39]. It is well known that the apparent diffusion coefficient (ADC) decreases during liver fibrosis, and hepatic ADC value is a good predictor of fibrosis stage^[40]. Recent studies showed that the ADC value is an effective non-invasive parameter for diagnosing and grading inflammation and liver fibrosis in children with chronic hepatitis, indicating that this method is of great significance in the diagnosis and classification of inflammation and liver fibrosis in children with chronic hepatitis^[41,42]. Moreover, splenic ADC value can predict esophageal varices in patients with liver cirrhosis and has a good correlation with laboratory biomarkers and clinical features of esophageal varices^[43], which provides new ideas for early diagnosis of esophageal varices in patients with chronic liver diseases.

Celecoxib is currently widely used in the clinical treatment of rheumatoid arthritis and osteoarthritis^[17]. Some researchers have shown that celecoxib can effectively improve portal hypertension and fibrosis in animal models. However, whether celecoxib relieves liver fibrosis by inhibiting ER stress still needs further exploration. This study proposed for the first time that celecoxib affects the progression of liver fibrosis by inhibiting ER stress, and we hope that this result can provide new targets for the treatment of liver fibrosis. However, multiple complex signaling pathways are regulated by ER stress, and whether other signals are involved in this process still needs further investigation. Second, when studying ER stress, it is necessary not only to investigate the related biochemical indicators, but also study it from a physiological perspective. Third, the imaging technology for liver fibrosis is developing rapidly, which has the advantages of high accuracy and little invasiveness. However, such technology was not utilized in the diagnosis of liver fibrosis in our animal model, and we should be in full consideration of it in our future work.

In conclusion, celecoxib reduces the severity of liver damage, improves the pathological changes of liver tissue, and has a beneficial effect on experimental liver fibrosis. Celecoxib treatment effectively reduces hepatocyte apoptosis in TAA-induced cirrhotic rats, which may be achieved by inhibiting the expression of CHOP after ER stress induction.

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis is a significant sign of chronic liver diseases, which could be treated and even reversed in the early stage. Inhibition of hepatocyte apoptosis is an important cause of reversal of liver fibrosis. Endoplasmic reticulum (ER) stress-mediated apoptosis is one of the important mechanisms of liver fibrosis. The cyclooxygenase-2 inhibitor celecoxib can improve the thioacetamide (TAA)-induced liver fibrosis in rats, and thus reverse the development of liver fibrosis. Whether celecoxib can inhibit apoptosis by inhibiting ER stress and further reverse liver fibrosis remains to be further studied.

Research motivation

Celecoxib has been widely used in the clinical treatment of rheumatoid arthritis and osteoarthritis. However, whether celecoxib can suppress apoptosis by inhibiting ER stress and further reverse liver fibrosis remains to be further studied.

Research objectives

This study aimed to explore the important role of celecoxib in modulating hepatocyte apoptosis during the development of liver fibrosis, and to clarify whether its regulatory mechanism is mediated by ER stress.

Research methods

Cirrhosis was induced by intraperitoneal injections of thioacetamide (TAA) for 16 wk (200 mg/kg per 3 d for the first 8 wk and 100 mg/kg per 3 d after 8 wk). Thirty-six male Sprague-Dawley rats were randomly divided into three groups: control, TAA, and TAA + celecoxib groups. In the last 8 wk, TAA-induced cirrhotic rats received celecoxib (20 mg/kg/day) or the vehicle by gastric gavage. After 16 wk, the rats were sacrificed, and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin (ALB) were detected. The hepatic fibrosis areas were evaluated by Sirius red staining and the degree of fibrosis was assessed by measuring the level of

hydroxyproline. ER stress levels were evaluated by detecting the marker proteins glucose-regulated protein 78 (GRP78), CCAAT/enhancer binding protein homologous protein (CHOP), PKR-like ER protein kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 alpha (IRE1α). Apoptosis levels were evaluated by detecting caspase-12 and caspase-3.

Research results

The serum ALT and AST levels in the liver were significantly reduced by celecoxib; however, the serum ALB had no significant changes. Celecoxib significantly reduced the degree of liver fibrosis and the levels of hydroxyproline (-38% and -25.7%, respectively, $P < 0.01$). Celecoxib ameliorated ER stress by reducing the level of GRP78 compared to the TAA group ($P < 0.05$). Consistently, after celecoxib administration, the upregulation of TAA-induced hepatic apoptosis markers (caspase-12 and caspase-3) and CHOP was significantly inhibited. In addition, after celecoxib treatment, the expression of key molecules associated with ER stress (PERK, ATF6, and IRE1) was decreased ($P < 0.05$).

Research conclusions

Therapeutic administration of celecoxib effectively reduces hepatic apoptosis in TAA-induced cirrhotic rats. The mechanism of action may be attributed to the suppression of CHOP expression, which subsequently inhibits ER stress.

Research perspectives

Our results indicate that celecoxib may play a role in inhibiting ER stress in rat liver fibrosis by inhibiting the expression of CHOP in the ER stress signaling pathway. Our data provide a new target for the treatment of liver fibrosis.

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Case Control Study

Food groups, diet quality and colorectal cancer risk in the Basque Country

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Abstract

BACKGROUND

The results obtained to date concerning food groups, diet quality and colorectal cancer (CRC) risk vary according to criteria used and the study populations.

AIM

To study the relationships between food groups, diet quality and CRC risk, in an adult population of the Basque Country (North of Spain).

METHODS

This observational study included 308 patients diagnosed with CRC and 308 age- and sex-matched subjects as controls. During recruitment, dietary, anthropometric, lifestyle, socioeconomic, demographic and health status information was collected. Adherence to the dietary recommendations was evaluated utilizing the Healthy Eating Index for the Spanish Diet and the MedDietScore. Conditional logistic regressions were used to evaluate the associations of food group intakes, diet quality scores, categorized in tertiles, with CRC risk.

RESULTS

The adjusted models for potential confounding factors showed a direct association between milk and dairy products consumption, in particular high-fat cheeses [odds ratio (OR) third tertile *vs* first tertile = 1.87, 95% confidence intervals (CI): 1.11-3.16], and CRC risk. While the consumption of fiber-containing foods, especially whole grains (OR third tertile *vs* first tertile = 0.62, 95%CI: 0.39-0.98), and fatty fish (OR third tertile *vs* first tertile = 0.53, 95%CI: 0.27-0.99) was associated with a lower risk for CRC. Moreover, higher MD adherence was associated with a reduced CRC risk in adjusted models (OR third tertile *vs* first tertile = 0.40, 95%CI: 0.20-0.80).

CONCLUSION

Direct associations were found for high-fat cheese, whereas an inverse relation was reported for fiber-containing foods and fatty fish, as well as adherence to a Mediterranean dietary pattern.

Key words: Colorectal cancer; Food group; Dietary quality; Mediterranean diet; Risk-factors; Case-control study

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Core tip: This matched case-control study supports the role of diet in colorectal cancer (CRC) risk. The results suggest that high consumption of high-fat cheeses is associated with CRC risk, whereas, a high intake of fiber-containing foods, especially whole grains, and fatty fish, as well as adherence to the Mediterranean dietary pattern, was associated with a lower risk for CRC. Future studies are needed to better understand the influence of the dietary habits on CRC prevention in this population that can provide leads for the design and tailoring of future interventions, and guide counselling strategies for promoting a healthy lifestyle.

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INTRODUCTION

Colorectal cancer (CRC) is a major public health challenge worldwide. CRC is the third-most commonly diagnosed malignancy and the fourth leading cause of cancer deaths in the world, accounting for approximately 1.8 million new cases and almost 900000 deaths in 2018^[1]. In Europe, CRC is the leading malignancy in terms of incidence and the second in mortality in both sexes^[2]. CRC is linked to western lifestyles, in particular, to diet, physical inactivity, smoking, alcohol consumption, and body weight^[3,4].

Epidemiological evidence suggests that dietary factors may both protect against and promote the development of CRC. A comprehensive review^[5] shows robust evidence about the protective role of dietary fiber. Other foods, such as milk or garlic, also may be protective. Conversely, red meat and processed meat intake and alcoholic drinks increase CRC risk. This food group approach has the advantage of reducing some of the problems inherent to analyses of nutrient intake (*e.g.*, inaccuracy and incompleteness of food-composition tables). Furthermore, it offers an advantage from a preventive perspective since food group results are easier to transform into dietary recommendations than those of nutrients^[6].

In this regard, foods are not consumed in isolation but as part of a dietary pattern; therefore, the actual effect of diet on disease risk may be observed only when all components are considered jointly^[6]. For this purpose, several diet quality indexes have been developed using point systems to measure whole diet quality based on the alignment of food choices with dietary recommendations. Some of these indices have been used to begin assessing the relationships between overall diet quality and CRC risk, and the results show that high scores in these indices are associated with a lower CRC risk^[7-10]. However, the results vary considerably according to the index used and other factors such as sex and age. Therefore, there is a need to further examine these relationships in diverse population studies.

The current case-control study was undertaken in the North of Spain to elucidate the relationships between food group consumption, diet quality and CRC risk, and identify possible differences in consumption depending on tumor location, in an adult population that participated in a CRC screening programme (CRCSP) in the Basque Country. To our knowledge, this is the first study in the Basque country population, in which both CRC incidence and mortality have increased in recent years^[11]. There are few studies in this regard in Spain^[12,13]. And both in these Spanish studies and in others carried out in other Mediterranean countries controls were apparently healthy subjects without clinical symptoms or signs of any type of cancer^[14].

MATERIALS AND METHODS

Study subjects

This is an observational, matched case-control study in a population group residing in the Basque Country (North of Spain). Participants in this study were recruited from among patients attending any of the three hospitals of the Osakidetza/Basque Health Service (Basurto, Galdakao and Donostia) members of the Basque Country CRCSP. To be eligible for this CRCSP, the patients had to be aged between 50 and 69, asymptomatic for colorectal symptoms and registered with the Osakidetza/Basque Health Service^[11]. These inclusion criteria were applied to both case and control group, that is, controls fulfilled the same eligibility criteria defined for the cases, with the exception of the disease (outcome). Recruitment and data collection for the present study were conducted between 2014 and 2016.

All the patients who were newly diagnosed with CRC ($n = 601$) were invited to participate in this study. Of those, 283 refused to participate in the study, and 10 were excluded due to missing information. Ultimately, 308 subjects (66.2% men) consented to participate in the survey and completed all the questionnaires. In addition, for each case, three age- (± 9.0 years) and sex-matched control patients were randomly sought from the list of CRC-free subjects ($n = 1836$) who participated in the CRCSP during the same period as the cases. The matched controls were patients with positive results

(abnormal) for immunochemical fecal occult blood test and negative colonoscopy results (normal). The participation rate of the controls was 37.6%, and 17 subjects were excluded due to missing information. Finally, the matched case-to-control ratio was 1:1, and the final data set included 308 cases who were diagnosed with CRC and 308 age- and sex-matched controls. Further details on recruitment and data collection have been described elsewhere^[15]. The main advantage of the present study compared to other above-mentioned researches^[12-14] is that we confirmed that controls were free of the disease through colonoscopy. Colonoscopy was used as diagnostic criteria to identify the cases in order to avoid false positives and negatives.

The pathological staging was based on the 7th edition of the AJCC cancer staging manual^[16] as follows: I (57.1%), IIA (13.6%), IIB (1.0%), IIC (0.3%), IIIA (7.5%), IIIB (14.6%), IIIC (1.9%), IVA (2.9%), and IVB (1.0%). The location of the cancer was distal in 76% and proximal (to the splenic flexure of the colon) in 24.0% of the samples. Concerning the tumor grade classification, we adopted a two-grade classification that was divided into low grade (well or moderately differentiated) (80.5%) and high grade (poorly differentiated, anaplastic, or undifferentiated) (4.5%); the percentage of missing data for this classification was 14.9%.

Some of the cases had undergone surgical resection (73.7%) and/or adjuvant treatments, chemotherapy (34.1%), and chemotherapy and radiation (6.8%). The percentages of subjects according to the type of surgical procedure were as follows: 26.3% sigmoidectomy, 17.5% right hemicolectomy resection, 18.8% low anterior resection, 6.5% left hemicolectomy resection, 2.3% transverse colectomy, 1.0% abdominoperineal resection, 1.0% total colectomy, and 0.3% transanal endoscopic operation. The cases were invited to take part in this survey at least one month after finishing their last treatment (surgery, chemotherapy or radiotherapy) (median, 1.3 years; range, 0.1 to 4.2 years). All the clinical data were obtained from the Basque Country's population-based CRCSP database, which links patient medical records and clinical databases and reviewed by expert staff. This review allowed the monitorization of all cases from the submission of the sample through the analysis, colonoscopy, pathology and follow-up.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving patients were approved by the Clinical Research Ethics Committee of the Basque Country (reference numbers PI2011006 and PI2014042). Written informed consent was obtained from all the study participants. Consenting participants self-completed and returned a detailed food frequency questionnaire (FFQ) and one general questionnaire. The questions referred to the behaviors before participating in the CRCSP. Assistance from the study staff was available to help the patients to understand the items on the questionnaires. The quality management applied in the present study has been described in a previous article^[15].

Dietary assessment

Diets were assessed using a short FFQ that was a modified version of the Rodríguez *et al.*^[17] (2008) questionnaire. This adaptation was validated with multiple 24-h recalls in the Basque general population^[18] and in CRC diagnosed patients in a pilot of the present study^[19]. It consists of 67 items and requires the subjects to recall the number of times each food item was consumed either per week or per month. This FFQ included specific questions about the frequency of intake of alcoholic beverages. Moreover, the respondents could also record the consumption of other foods that were not included on the food list.

Consumption frequencies were standardized to “per day” and multiplied by standard serving sizes (grams)^[20]. For items that included several foods, each food's contribution was estimated with weighting coefficients that were obtained from the usual consumption data^[21]. Food items were then regrouped according to nutritional characteristics^[22] and considering the potential contribution of food to the pathogenesis of CRC^[23,24]. Details on the items included in each food group are shown in [Table 1](#). All food items that were consumed were entered into DIAL 2.12 (2011 ALCE INGENIERIA)^[25], a type of dietary assessment software, to estimate energy intake (kilocalories/day, kcal/d).

Adherence to the dietary recommendations was evaluated utilizing the Healthy Eating Index for Spanish Diet (HEISD)^[26] and the MedDietScore (MDS)^[27], as previously described^[19]. The theoretical range of the HEISD is 0-100 and of the MDS 0-55, higher values of these scores indicate greater adherence to the dietary recommendations for the Spanish population and the Mediterranean diet pattern, respectively. HEISD was divided into the following categories: Poor diet (< 50 points), needs improvement (50-80 points) and proper diet (> 80 points)^[26]; and the MDS into

Table 1 Food group definitions

Food group	Food items
Red and processed meat	
Red meat	Beef, pork and lamb, minced meat, hamburgers, meatballs
Processed meat	Ham, sausage, salami, mortadella, black pudding or blood sausage
Egg	Egg
Fish	White fish (hake, grouper, sole, cod) and fatty fish (sardine, tuna, salmon, mackerel)
Milk/ dairy products	
Non-cheese products	Whole milk, semi-skimmed milk, skimmed milk, whole yogurt, skimmed yogurt and dairy desserts
Cheese	Burgos cheese, curd, cottage and cheeses low in calories, mature, semi-mature and creamy cheese
Fiber-containing foods	
Fruits	Orange, tangerine, apple, pear, banana, peach, raisins, prunes, dried figs... natural fruit juices
Vegetables	Salads, green beans, chard, spinach... garnish vegetables (eggplant, mushrooms, peppers...), garlic, onion
Whole grains	Whole grain pasta, brown rice, whole grain cookies, whole breakfast cereals (Muesli, All-Bran)
Nuts	Walnuts, almonds, hazelnuts, peanuts
Fat	Vegetable oils (olive, sunflower, corn, soy), butter, margarine, mayonnaise
Sweet and added sugar	Chocolate, breakfast cereals, cookies, muffins, donuts, honey, sugar, commercial fruit juice, soft-drinks, cakes, pies
Alcoholic beverages	Beer, wine, hard cider, vermouth, whiskey, rum, gin, brandy, cocktails

the following ones: Low adherence to MD (0-34 points) and high adherence (> 35 points). The cut-off point of MDS was established taking into account that scores below 34 points were associated with a higher risk of coronary heart disease, being the relative odds $\geq 1.42^{[27]}$.

General questionnaire

A general questionnaire was used to gather information on weight status (self-reported weight and height) and environmental factors [demographic factors: Age and sex; and lifestyle information: Physical exercise (PE) and smoking consumption]. These questions were taken from the Spanish Health Questionnaire^[28]. Body mass index (BMI) estimated from self-reported height and weight was classified according to the World Health Organization criteria for those under 65 years of age^[29] and according to the criteria proposed by Silva Silva Rodrigues *et al*^[30] for those 65 years and older.

Additionally, socioeconomic and health status data were assessed with two indices that were obtained from the clinical databases developed by the Health Department of the Basque Government, namely the socioeconomic deprivation index (DI) and predictive risk modelling (PRM), respectively. The first one was estimated using the MEDEA project criteria^[31], as has been described elsewhere^[12] and was divided into quintiles (Q), with the first being the least disadvantaged and the fifth being the most disadvantaged. The DI was successfully assigned to 80.2% of participants, while the quality of the registered information did not permit the linking of the remaining 19.8%.

The PRM is an index that is based on Adjusted Clinical Groups^[32], Diagnostic Cost Groups/Hierarchical Condition Categories^[33] and Clinical Risk Groups^[34]. This index combines information about diagnoses, prescriptions, previous costs and the use of specific procedures. It is capable of predicting the use of health resources^[35], and it was stratified into four levels (L); the first included participants with a risk of high health resource consumption and the fourth included those with low health resource consumption. The PRM was successfully assigned to 95.1% of participants, while the quality of the registered information did not permit the linking of the remaining 4.9%.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, United States) and STATA 13.0 (StataCorp LP, TX, United States). Categorical variables are shown as a percentage, and continuous variables are shown as the means and standard deviations (SD). Normality was

checked using the Kolmogorov-Smirnov-Lilliefors test. Differences between continuous variables were calculated with a Wilcoxon test, and a McNemar's test was used for categorical variables.

Conditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (95%CI) for CRC risk according to tertiles of food group intakes and diet quality scores for unadjusted and adjusted models. Intake of all food groups and total diet quality scores were categorized into tertiles by the distribution in the control population, taking into account sex differences when they were significant. The lowest tertile was used as the reference group. Tertile cut-offs for HEISD were: 1st tertile (T_1), 69; 2nd tertile (T_2), 69-74.5 and 3rd tertile (T_3), > 74.5; and for MDS: T_1 , < 35; T_2 , 35-37 and T_3 , > 37.

Based on known risk factors for CRC^[36-38], covariates in adjusted models included age, sex, weight status, energy intake, PE level, smoking status, intensity of smoking (in current and past smokers) and time not smoking (in past smokers), DI and PRM. Quantitative covariates (cigarettes/d and years not smoking) were dichotomized by mean or median, according to the normality test. We used the cut-off of Romaguera *et al.*^[39] to create two PE levels expressed in min/d of cycling/sports: Sedentary-light (< 15 min/d) and moderate-vigorous (≥ 15 min/d). Age was dichotomized using the same age ranges that were used in the sample selection process (50-59 years old *vs* 60-69 years old). Qualitative ones, such as DI and PRM were dichotomized taking into account the distribution of frequencies to obtain similar sample sizes for each category (DI, Q_{1-3} *vs* Q_{4-5} ; PRM, L_{3-4} *vs* L_{1-2}). Energy intake was included as a quantitative variable in the adjusted models. We included participants with missing data for the covariates as a separate category. The reference categories were those that, according to the literature, have a lower CRC risk. All tests were 2-sided, and *P* values less than 0.05 were considered statistically significant.

RESULTS

Comparisons of general characteristics between the cases and the controls are presented in Table 2. Significant differences between the cases and the controls were found for educational level, smoking and weight status, with a higher percentage of cases with low-medium educational level, past or current smoking status and with overweight/obesity compared to the controls ($P < 0.01$).

Table 3 shows food group intakes expressed as mean values and standard deviations according to case-control status. No significant differences were found between the two groups for the majority of foods groups, except for a higher consumption of eggs and a lower intake of whole grains in the cases than the controls ($P < 0.05$).

The ORs for CRC risk by the main food group and food subgroup intakes are presented in Table 4 and 5, respectively. The adjusted ORs for CRC risk increased with higher red and processed meat, eggs, milk/dairy products intakes; whereas it decreased with higher fiber-containing foods and nut intakes. The food group with the highest adjusted OR for CRC risk was milk/dairy products. Fish consumption showed an association with CRC risk in the unadjusted analysis but not in the adjusted analysis. For some of these food groups, specifically for red and processed meat, fish, eggs and nuts, the null value 1 was contained in the confidence interval. Concerning the food subgroup intakes, the ORs for CRC risk increased with higher high-fat cheese intakes, while it decreased with higher fatty fish, in the adjusted analysis.

Supplementary Table 1 describes food group intakes of cases according to tumor location and their matched controls. Food group intakes were not substantially different between proximal and distal cancer cases, except for fish, milk/dairy products and fat. The fish consumption was higher in both case subgroups (proximal and distal cancer cases) in comparison with their matched controls ($P < 0.001$). However, the milk/dairy products intake was higher in proximal tumor cases and was lower in distal tumor cases than in their matched controls ($P < 0.001$). Finally, the fat intake was higher in proximal tumor cases in comparison with their matched controls ($P < 0.05$). The sample sizes did not allow the assessment of food group intakes related to disease risk, stratifying according to the tumor location.

The components and total scores of the HEISD and MDS are displayed in Table 6. According to HEISD, 91.9% of the participants (cases and controls) followed a diet classified as "needs improvement", 7.6% followed a "good diet" and 0.5 followed a "poor diet". Significant differences were neither observed in the HEISD classification nor the components scores nor in the total score. However, the total score for this

Table 2 General characteristics of the sample studied

Characteristics	Cases (n = 308)		Controls (n = 308)		P value
Sex, men, n (%)	204 (66.2)		204 (66.2)		
Age, yr, mean SD	61.5	5.2	61.1	5.5	0.093
Schooling, %					
No education/primary education	36.7		29.2		
Technical/secondary education	48.0		44.5		
University degree	15.3		26.3		0.005
Economic activity, %					
Working	27.9		32.1		
Unemployed	5.2		3.2		
Retired	58.8		56.2		
Housework	8.1		8.4		0.496
Last work, %					
Employer or businessman/women	19.2		17.9		
Steady salaried employee	75.0		71.8		
Temporary salaried employee or member of a cooperative	0.6		4.5		
Household help and other activities without salary	5.1		5.8		0.073
Smoking status, %					
Never	27.9		38.6		
Past/current	72.1		61.4		0.004
Time to quit smoking					
≥ 11 yr	67.2		66.7		
< 11 yr	32.8		33.3		0.931
Intensity of smoking ¹					
≤ 15 cigarettes/d	50.7		33.1		
> 15 cigarettes/d	49.3		66.9		0.003
Physical exercise, %					
< 15 min/d of cycling/sports	79.2		65.9		
≥ 15 min/d of cycling/sports	20.8		34.1		< 0.001
BMI, %					
Underweight	6.5		7.8		0.033
Normal weight	26.0		34.1		
Overweight/obesity	67.5		58.1		
Energy intake (kcal/d), mean SD	1769.9	383.4	1736.6	388.2	0.172
DI, % ²					
Q ₁₋₃	47.1		65.6		
Q ₄₋₅	18.8		29.5		< 0.001
PRM, % ²					
L ₁₋₂	15.6		12.3		
L ₃₋₄	83.4		79.2		< 0.001

¹Percentages were calculated excluding never smokers.

²Valid percentages. BMI: Body mass index; DI: Deprivation index (This index was successfully assigned to 80.2% of the study sample); L: Level; PRM: Predictive risk modelling (This index was successfully assigned to 95.1% of the study sample); Q: Quintile; SD: Standard deviation.

Table 3 Food group intakes of the sample studied

Food groups, g/d	Cases (n = 308)		Controls (n = 308)		P value
	mean	SD	mean	SD	
Red and processed meat	70.9	36.6	66.0	39.7	0.064
Red meat	49.7	30.5	46.1	31.0	0.130
Processed meat	21.2	16.6	19.9	17.2	0.155
Total fish	76.8	39.2	77.6	40.9	0.540
White fish	40.9	25.2	44.1	27.4	0.055
Fatty fish	35.9	22.6	33.6	24.1	0.236
Eggs	20.8	12.7	18.7	11.5	0.038
Milk/dairy products	264.7	153.4	271.0	119.4	0.310
Non-cheese dairy products	246.0	152.4	253.8	118.5	0.203
Total cheeses	18.8	17.4	17.1	16.8	0.172
Fresh cheeses ¹	6.9	10.3	7.1	13.3	0.867
Other cheeses ²	11.7	11.8	10.1	10.7	0.172
Fiber-containing foods	570.3	243.9	564.8	214.1	0.761
Fruits (including natural juices)	330.2	202.5	322.6	168.2	0.791
Vegetables	202.1	88.8	200.6	90.9	0.803
Whole grains	14.4	19.9	18.8	23.4	0.012
Fat	35.5	6.9	34.6	6.4	0.064
Nuts	9.1	10.1	10.9	10.5	0.055
Sweets and added sugar	108.3	95.4	110.7	116.5	0.969
Alcoholic beverages	103.4	100.7	96.8	105.9	0.269

¹Fresh cheeses, *e.g.*, Burgos cheese and cheeses low in calories.

²Other cheeses, mature, semi-mature and creamy cheeses. SD: Standard deviation.

dietary quality index and the score of diet variety components were higher for cases with the proximal location of cancer than for their matched controls ($P < 0.05$) (Supplementary Table 2). No association was found between this index and risk of CRC, in the conditional logistic regressions.

Concerning the MDS, in the total sample, 39.8% showed low adherence to the MD and the remaining percentage had high adherence, without significant differences in the MDS classification between the cases and the controls. However, the scores for whole grains and total index were lower for cases than for controls ($P < 0.05$). This last result was confirmed using conditional logistic regressions, showing that those participants with higher MDS had a lower CRC risk than those with a lower score, in both unadjusted (model I: T_3 vs T_1 , OR = 0.57, 95%CI: 0.37-0.89, $P = 0.013$) and adjusted models (model II: T_3 vs T_1 , OR = 0.40, 95%CI: 0.20-0.80, $P = 0.009$). No significant differences were observed in total MDS between cases stratified by tumor location and their matched controls, but the total score was higher for cases with the proximal location of cancer than for those with distal location ($P < 0.05$) (Supplementary Table 2). Moreover, the score for potatoes and whole grain components were lower for cases with the distal location of cancer than for their matched controls ($P < 0.05$).

Table 4 Association between main food group intake and colorectal cancer risk

Main food group intakes ¹	No., case/control	Model I ²	Model II ³	Model III ⁴
		OR (95%CI)	OR (95%CI)	OR (95%CI)
Red and processed meat				
T ₁	90/102	1.00	1.00	1.00
T ₂	97/103	1.06 (0.72-1.57)	1.02 (0.61-1.72)	1.08 (0.61-1.94)
T ₃	121/109	1.32 (0.91-1.93)	1.65 (0.99-2.75)	1.26 (0.71-2.23)
P		0.314	< 0.001	-
Fish				
T ₁	95/105	1.00	1.00	1.00
T ₂	77/103	0.82 (0.53-1.25)	0.97 (0.56-1.68)	0.83 (0.46-1.51)
T ₃	136/105	1.49 (1.01-2.20)	1.06 (0.62-1.79)	1.25 (0.68-2.29)
P		0.008	< 0.001	-
Eggs				
T ₁	71/98	1.00	1.00	1.00
T ₂	107/116	1.15 (0.77-1.72)	1.04 (0.62-1.76)	0.97 (0.61-1.93)
T ₃	130/104	1.55 (1.03-2.33)	1.72 (1.00-2.94)	1.26 (0.71-2.23)
P		0.081	< 0.001	-
Milk/dairy products				
T ₁	60/102	1.00	1.00	1.00
T ₂	127/104	2.05 (1.35-3.11)	2.02 (1.19-3.42)	1.97 (1.10-3.53)
T ₃	121/102	2.00 (1.31-3.05)	2.12 (1.25-3.84)	1.80 (0.95-3.42)
P		< 0.001	< 0.001	-
Fiber-containing foods				
T ₁	121/102	1.00	1.00	1.00
T ₂	75/101	0.60 (0.39-0.92)	0.47 (0.26-0.85)	0.49 (0.25-0.95)
T ₃	112/105	0.86 (0.58-1.28)	0.63 (0.36-1.11)	0.65 (0.35-1.21)
P		0.048	< 0.001	-
Nuts				
T ₁	118/103	1.00	1.00	1.00
T ₂	118/108	0.97 (0.67-1.42)	0.87 (0.53-1.44)	0.70 (0.37-1.31)
T3	72/97	0.67 (0.43-0.97)	0.58 (0.34-1.00)	0.59 (0.30-1.18)
P		0.074	< 0.001	-
Fat				
T ₁	86/100	1.00	1.00	1.00
T ₂	101/105	1.12 (0.75-1.67)	0.94 (0.56-1.59)	0.83 (0.45-1.51)
T ₃	121/100	1.34 (0.92-1.97)	1.46 (0.85-2.50)	1.25 (0.68-2.29)
P		0.297	< 0.001	-
Sweets and added sugar				
T ₁	82/120	1.00	1.00	1.00
T ₂	120/103	1.47 (0.99-2.20)	1.67 (0.98-2.86)	1.88 (1.01-3.52)
T ₃	106/103	1.30 (0.87-1.94)	1.63 (0.92-2.89)	1.39 (0.72-2.67)

<i>P</i>		0.159	< 0.001	-
Alcoholic beverage				
T ₁	90/103	1.00	1.00	1.00
T ₂	107/101	1.20 (0.81-1.77)	1.05 (0.63-1.75)	1.10 (0.63-1.92)
T ₃	111/104	1.19 (0.83-1.72)	0.82 (0.50-1.36)	0.75 (0.42-1.32)
<i>P</i>		0.558	< 0.001	-

¹Food groups consumption was categorized into tertiles according to the distribution in controls, and by sexes for food groups with significant differences according to sex; Tertiles of food groups: Red and processed meat, T₁ < 47.7, T₂ 47.7-78.5, T₃ > 78.5; Total fish, T₁ < 42.8, T₂ 42.8-67.2, T₃ > 67.2; eggs, T₁ < 15.7, T₂ 15.7-23.5, T₃ > 23.5; Milk/dairy products, T₁ < 72.0, T₂ 72.0-232.1, T₃ > 232.1; Fat, T₁ < 30.8, T₂ 30.8-34.8, T₃ > 34.8; Nuts, T₁ < 2.9, T₂ 2.9-12.8, T₃ > 12.8; Sweets and added sugar, T₁ < 50.1, T₂ 50.1-117.3, T₃ > 117.3; Tertiles of food groups for men: Fiber-containing foods, T₁ < 424.3, T₂ 424.3-617.8, T₃ > 617.8; Alcoholic beverages, T₁ < 66.7, T₂ 66.7-137.2, T₃ > 137.2; Tertiles of food groups for women: Fiber-containing foods, T₁ < 537.9, T₂ 537.9-723.6, T₃ > 723.6; T₁ < 8.3; T₂ 8.3-85.7; T₃ > 85.7.

²Model I, analyses were performed using crude conditional logistic regression, without taking into account confounding factors.

³Model II, analyses were performed using conditional logistic regression analysis adjusted for age (50-59 years old, 60-69 years old), sex, body mass index (underweight/normal weight, overweight/obesity), energy intake (kcal/d), physical exercise level (< 15 min/d of cycling/sports, ≥ 15 min/d), smoking status and intensity of smoking (never; past: quit smoking ≥ 11 years ago, quit < 11 years ago; Smoker: ≤ 15 cigarettes/d, > 15 cigarettes/d), Deprivation Index (quintile 1-3, quintile 4-5) and Predictive Risk Modelling (level 1-2, level 3-4), including food groups separately; participants with missing data for the confounding variables were included as a separate category for these variables.

⁴Model III, model II including all the mean food groups. CI: Confidence interval; OR: Odd ratio; T: Tertile.

DISCUSSION

The results from this observational study indicate that high consumption of milk/dairy products, in particular high-fat cheeses, is associated with CRC risk, while a high intake of fiber-containing foods, specially whole grains, and fatty fish was associated with a lower risk for CRC. Moreover, a higher MD adherence in general and particularly a higher score for whole grains have been associated with a reduced CRC risk.

As other authors have previously reported^[40] milk/dairy products were the food group with the highest adjusted OR for CRC risk, which is not in agreement with the probable evidence of protection of this food group against CRC^[5]. Some cohort studies support the protective effect of total dairy products and milk^[41-43]. This effect has been hypothetically associated with calcium, vitamin D, fats and other components such as lactoferrin or lactic bacteria in the case of fermented dairy products milk^[41,42]. However, case-control studies published to date are heterogeneous and, on average, do not provide evidence of an association between total intake of total dairy products, milk, cheese or yogurt and CRC risk^[41]. Regarding milk/dairy products consumption according to anatomical subsites of cases, the intake was higher in proximal tumor cases and lower in distal cases than in their matched controls. Although according to scientific literature, the effect of this food group seems to be similar across all locations of the bowel^[43].

In general, epidemiological studies have not found evidence of either reduction or increase of CRC risk specifically associated with the consumption of cheese^[41,42]. Although there are few pieces of research on cheese consumption that reported an inverse association with CRC^[44] in the present research, high-fat cheeses are shown to be possible risk factors for CRC development. Some studies showed a positive relationship between fatty foods and CRC incidence^[45]. Dairy products, *e.g.*, mature, semi-mature and creamy cheeses, are rich in saturated fat, so this relationship might be due to the content of fat in these products. Several studies have suggested that high-fat consumption increases bile acid discharge. Moreover, an increase in the concentration of bile acids above physiological levels has been reported to promote CRC^[46,47]. In any case, the association between milk/dairy products consumption and the risk of developing CRC is complex and some researchers indicated that the fat content contained within dairy products does not influence this association^[43].

In line with previous studies^[48-50], we also found that the consumption of fiber-containing foods was inversely associated with CRC risk. Specifically, consumption of more than 424.3 g/d in men and 537.9 g/d in women of fiber-containing foods decreased CRC risk by about 50% (OR approximately 0.5) compared to lower consumption, in adjusted models. The preventive effect of dietary fiber can be explained by biological mechanisms that include increasing amounts of feces, decreasing gastrointestinal transit time, diluting intestinal cancer-causing factors,

Table 5 Association between food subgroup intakes and colorectal cancer risk

Food subgroup intakes ¹	No., case/control	Model I ²	Model II ³	Model III ⁴
		OR (95%CI)	OR (95%CI)	OR (95%CI)
Red meat				
T ₁	88/101	1.00	1.00	1.00
T ₂	103/98	1.20 (0.81-1.79)	1.38 (0.82-2.34)	1.10 (0.62-1.96)
T ₃	117/109	1.22 (0.84-1.78)	1.41 (0.87-2.30)	1.17 (0.67-2.03)
P		0.534	< 0.001	-
Processed meat				
T ₁	102/103	1.00	1.00	1.00
T ₂	82/99	0.84 (0.57-1.24)	0.62 (0.36-1.07)	0.67 (0.38-1.18)
T ₃	124/106	1.21 (0.83-1.77)	1.54 (0.91-2.60)	1.54 (0.88-2.70)
P		0.206	< 0.001	-
White fish				
T ₁	95/105	1.00	1.00	1.00
T ₂	77/103	0.82 (0.53-1.25)	0.97 (0.56-1.68)	0.96 (0.36-2.53)
T ₃	136/105	1.49 (1.01-2.20)	1.06 (0.62-1.79)	1.29 (0.74-2.25)
P		0.008	< 0.001	-
Fatty fish				
T ₁	119/110	1.00	1.00	1.00
T ₂	105/102	1.05 (0.71-1.55)	0.93 (0.56-1.55)	0.89 (0.43-1.69)
T ₃	74/96	0.72 (0.49-1.08)	0.50 (0.29-0.87)	0.53 (0.27-0.99)
P		0.145	< 0.001	-
Fresh cheese				
T ₁	150/153	1.00	1.00	1.00
T ₂	224/33	0.64 (0.32-1.28)	1.06 (0.44-2.55)	1.11 (0.66-1.87)
T ₃	134/122	1.11 (0.80-1.55)	1.10 (0.70-1.72)	0.92 (0.58-1.46)
P		0.272	< 0.001	-
Other cheeses				
T ₁	96/116	1.00	1.00	1.00
T ₂	71/75	1.16 (0.76-1.77)	1.51 (0.86-2.63)	1.83 (1.15-2.89)
T ₃	141/117	1.46 (1.01-2.12)	1.85 (1.12-3.05)	1.87 (1.11-3.16)
P		0.112	< 0.001	-
Fruits				
T ₁	109/99	1.00	1.00	1.00
T ₂	98/110	0.82 (0.56-1.19)	1.08 (0.63-1.85)	1.03 (0.58-1.83)
T ₃	101/99	0.92 (0.62-1.37)	0.70 (0.40-1.22)	0.68 (0.37-1.26)
P		0.567	< 0.001	-
Vegetables				
T ₁	97/102	1.00	1.00	1.00
T ₂	111/103	1.14 (0.76-1.71)	0.98 (0.55-1.73)	1.10 (0.60-2.04)
T ₃	100/103	1.03 (0.68-1.57)	0.94 (0.52-1.70)	1.10 (0.58-2.11)

<i>P</i>		0.789	< 0.001	-
Whole grains				
T ₁	144/128	1.00	1.00	1.00
T ₂	83/77	0.92 (0.62-1.38)	0.86 (0.52-1.42)	0.98 (0.58-1.65)
T ₃	81/103	0.68 (0.46-1.01)	0.62 (0.37-1.06)	0.62 (0.39-0.98)
<i>P</i>		0.135	< 0.001	

¹Food groups consumption was categorized into tertiles according to the distribution in controls, and by sexes for food groups with significant differences according to sex; Tertiles of food groups: Red meat, T₁ < 33.5, T₂ 33.5-54.9, T₃ > 54.9; Processed meat, T₁ < 11.6, T₂ 11.6-22.8, T₃ > 22.8; non-cheese dairy, T₁ < 225.0, T₂ 225.0-325.0, T₃ > 325.0; Cheese, T₁ < 7.5, T₂ 7.5-20.0, T₃ > 20.0; Vegetables, T₁ < 152.9, T₂ 152.9-237.2, T₃ > 237.2; Tertiles of food groups for men: Fruits, T₁ < 207.5, T₂ 207.5-392.9, T₃ > 392.9; Whole grains, T₁ < 1.0, T₂ 1.0-17.5, T₃ > 17.5; Tertiles of food groups for women: Fruits T₁ < 242.9, T₂ 242.9-425.0; Whole grains, T₁ < 2.0, T₂ 2.0-30.0, T₃ > 30.0.

²Model I, analyses were performed using crude conditional logistic regression, without taking into account confounding factors.

³Model II, analyses were performed using conditional logistic regression analysis adjusted for age (50-59 years old, 60-69 years old), sex, body mass index (underweight/normal weight, overweight/obesity), energy intake (kcal/d), physical exercise level (< 15 min/d of cycling/sports, ≥ 15 min/d), smoking status and intensity of smoking (never; past: quit smoking ≥ 11 years ago, quit < 11 years ago; Smoker: ≤ 15 cigarettes/d, > 15 cigarettes/d), Deprivation Index (quintile 1-3, quintile 4-5) and Predictive Risk Modelling (level 1-2, level 3-4), including food groups separately; Participants with missing data for the confounding variables were included as a separate category for these variables.

⁴Model III, model II including all the mean food groups. CI: Confidence interval; OR: Odd ratio; T: Tertile.

interfering absorption of those, and lowering intestinal acidity^[51]. In addition, fermentation of fiber produced butyrate. This short-chain fatty acid showed anti-inflammatory, anti-proliferation and antineoplastic properties in colonocyte cells metabolism through microbiota homeostasis and genetic/epigenetic regulation^[52].

Furthermore, our findings suggest that high consumption of whole grains (higher than 17.5 g/d in men and 30.0 g/d in women) may decrease the risk of CRC, after controlling confounding factors. There is convincing evidence that whole grains help to reduce CRC risk^[5,53]. The observed reduction in CRC risk associated with high consumption of whole grains may partly be attributed to dietary fiber, resistant starch, and oligosaccharides that can influence the gut environment. Insoluble fiber increases the bulk of luminal contents, diluting potential carcinogens and promoters in the colon and decreasing transit time, and, consequently, reduces the exposure of the colonic epithelium to harmful compounds^[54,55]. Additionally, other components such as vitamins (especially B-vitamins), minerals (*e.g.*, magnesium and zinc), phenolic compounds, antinutrients (*e.g.*, tannins), and phytoestrogens may also contribute to this protection^[54].

On the other hand, the consumption of fatty fish (higher than 42.8 g/d) was associated with a decreased risk in CRC by about 50% (OR approximately 0.5) compared to lower consumption, after adjusting models for covariates. It should be noted that the Basque Country population has a higher consumption of total fish and fatty fish compared to other Spanish autonomous communities^[55,56]. Recent cohort studies have observed that fatty fish was inversely associated with CRC incidence^[57,58] and they have related this association with exposure to long-chain n-3 polyunsaturated fatty acids^[57]. Evidence from animal and *in vitro* studies indicates that n-3 fatty acids present in fatty fish may inhibit carcinogenesis^[59]. High intake of n-3 fatty acids suppresses the production of arachidonic acid-derived eicosanoids such as prostaglandin E2 and leukotriene B431. N-3 fatty acids could also suppress the expression of inducible nitric oxide synthase and nuclear transcription factor κ B (NF-κ B)^[60].

In relation to the diet quality, our findings on the MDS and CRC risk are supported by those of other researchers^[13,61-63], who found significant associations between lower risk of CRC and adherence to Mediterranean dietary pattern. However the HEISD was not associated with CRC risk, discrepancies in results obtained with the two dietary quality indices analysed are probably due to differences in their constructs and scoring criteria. The overall MDS was inversely associated with CRC risk, being higher the total score in cases with the proximal location of cancer than for those with the distal location. These last results contrast with previous findings, which showed that the protective effects of adherence to the MD were mainly for distal colon and rectal cancer and not for proximal colon cancer^[64]. In the total sample, investigation of the separate score components showed that whole grain score was lower for cases than for controls. This result is consistent with that obtained for the association between whole grains consumption and CRC risk.

Table 6 Diet quality indices in the sample studied

	Cases (<i>n</i> = 308)		Controls (<i>n</i> = 308)		<i>P</i> value
	mean	SD	mean	SD	
HEISD components ¹					
Meats	3.1	1.7	3.1	1.7	0.811
Processed meats	2.8	2.0	3.1	2.2	0.162
Legumes	8.6	2.1	8.5	2.3	0.716
Milk/Dairy	9.8	1.2	9.8	1.1	0.797
Fruits	9.1	1.8	9.1	1.9	0.464
Vegetables	8.9	1.7	8.9	1.6	0.816
Grains	9.9	0.9	9.9	1.9	0.862
Sweets	1.6	3.1	1.6	3.0	0.847
Soft-drink	8.8	2.5	8.7	2.6	0.583
Variety	8.0	1.7	8.0	1.7	0.646
Total HEISD	70.7	7.2	70.8	7.9	0.906
MDS components ²					
Red meats and processed meats	0.7	1.0	0.8	1.2	0.134
Poultry	2.9	1.2	2.9	1.2	0.335
Fish	3.8	1.1	3.8	1.2	0.771
Legumes	2.4	1.2	2.3	1.1	0.482
Full fat dairy	2.0	1.8	2.0	1.9	0.618
Vegetables	4.9	0.6	4.9	0.5	0.599
Fruits	4.6	1.0	4.6	1.0	0.726
Potatoes	2.2	1.5	2.4	1.5	0.054
Whole grains	2.0	2.2	2.3	2.3	0.044
Alcoholic beverages	4.9	0.4	4.9	0.4	0.729
Olive oil	4.9	0.6	4.8	0.8	0.446
Total MDS	35.3	4.5	36.0	4.3	0.027

¹Each component can contribute 10 points to the total score and the theoretical range is 0–100.

²Each component can contribute five points to the total score and the theoretical range is 0–55. HEISD: Healthy Eating Index for Spanish Diet; MDS: Med Diet Score; SD: Standard deviation.

Our study has several limitations. First, recall bias inherent in a case-control study design cannot be ruled out. The primary concern of this study is the low participation rate, which may have limited the representativeness of study samples. The decision to participate or not may be influenced by several factors, including social, educational and health conditions, which may again correlate with outcome risk factors. Second, self-reported data could be subject to measurement errors and the problem of food omissions due to memory failure and underreporting of unhealthy habits among disease subjects. However, previous validation studies indicate that the self-reported dietary information is reported with sufficient accuracy for use in epidemiology analyses^[65]; and it should be noted that dietary changes are usually modest after participating in the CRCSP due to a lack of information and personalized advice^[66,67]. Another limitation of this type of study could be the selection of controls (selection bias). To avoid this type of bias, we obtained controls from the same CRCSP and in the same period as cases, thus, it was confirmed that they did not suffer from CRC by colonoscopy.

Despite these limitations, the results allow us to conclude that high consumption of high-fat cheeses is associated with CRC risk, whereas, a high intake of fiber-containing

foods, especially whole grains, and fatty fish, and adherence to the Mediterranean dietary pattern was associated with a lower risk for CRC. Future studies are needed to better understand the influence of the dietary habits on CRC prevention in this population that can provide leads for the design and tailoring of future interventions, and guide counselling strategies for promoting a healthy lifestyle.

ARTICLE HIGHLIGHTS

Research background

Epidemiological evidence suggests that some foods may both protect against and promote the development of colorectal cancer (CRC). However, foods are not consumed in isolation but as part of a dietary pattern; therefore, the actual effect of diet on disease risk may be observed only when all components are considered jointly. For this purpose, several diet quality indexes have been developed using point systems to measure whole diet quality based on the alignment of food choices with dietary recommendations.

Research motivation

Some diet quality indexes have been used to begin assessing the relationships between overall diet quality and CRC risk, and the results show that high scores in these indices are associated with a lower CRC risk. However, the results vary considerably according to the index used and other factors such as sex and age. Therefore, there is a need to further examine these relationships in diverse population studies.

Research objectives

To study the relationships between food groups, diet quality and CRC risk, in an adult population of the Basque Country (North of Spain).

Research methods

This observational study included 308 patients diagnosed with CRC and 308 age- and sex-matched subjects as controls. During recruitment, dietary, anthropometric, lifestyle, socioeconomic, demographic and health status information was collected. Dietary intake was assessed using a short food frequency questionnaire that was adapted and validated for this population. Adherence to the dietary recommendations was evaluated utilizing the Healthy Eating Index for the Spanish Diet and the MedDietScore. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, United States) and STATA 13.0 (StataCorp LP, TX, United States). Conditional logistic regressions were used to evaluate the associations of food group intakes, diet quality scores, categorized in tertiles, with CRC risk.

Research results

The adjusted models for potential confounding factors showed a direct association between milk/dairy products consumption, in particular high-fat cheeses [odds ratio (OR) third tertile *vs* first tertile = 1.87, 95% confidence intervals (CI): 1.11-3.16], and CRC risk. While the consumption of fiber-containing foods, especially whole grains (OR third tertile *vs* first tertile = 0.62, 95%CI: 0.39-0.98), and fatty fish (OR = 0.53, 95%CI: 0.27-0.99) was associated with a lower risk for CRC. Moreover, higher MD adherence was associated with a reduced CRC risk in adjusted models (OR = 0.40, 95%CI: 0.20-0.80).

Research conclusions

Direct associations were found for high-fat cheese, whereas an inverse relation was reported for fiber-containing foods and fatty fish, as well as adherence to a Mediterranean dietary pattern.

Research perspectives

Future studies are needed to better understand the influence of the dietary habits on CRC prevention in this population that can provide leads for the design and tailoring of future interventions, and guide counselling strategies for promoting a healthy lifestyle.

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Retrospective Study

Primary sclerosing cholangitis associated colitis: Characterization of clinical, histologic features, and their associations with liver transplantation

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Abstract

BACKGROUND

Primary sclerosing cholangitis (PSC) associated inflammatory bowel disease (IBD) is a unique form of IBD (PSC-IBD) with distinct clinical and histologic features from ulcerative colitis (UC) and Crohn disease (CD). In patients with PSC and IBD, the severity of the two disease processes may depend on each other.

AIM

To study the histologic and clinical features of PSC patients with and without IBD.

METHODS

We assessed specimens from patients with UC ($n = 28$), CD ($n = 10$), PSC and UC (PSC-UC; $n = 26$); PSC and CD (PSC-CD; $n = 6$); and PSC and no IBD (PSC-no IBD; $n = 4$) between years 1999-2013. PSC-IBD patients were matched to IBD patients without PSC by age and colitis duration. Clinical data including age, gender, age at IBD and PSC diagnoses, IBD duration, treatment, follow-up, orthotopic liver transplantation (OLT) were noted.

RESULTS

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PSC-UC patients had more isolated right-sided disease ($P = 0.03$), and less active inflammation in left colon, rectum ($P = 0.03$ and $P = 0.0006$), and overall ($P = 0.0005$) compared to UC. They required less steroids ($P = 0.01$) and fewer colectomies ($P = 0.03$) than UC patients. The PSC-CD patients had more ileitis and less rectal involvement compared to PSC-UC and CD. No PSC-CD patients required OLT compared to 38% of PSC-UC ($P = 0.1$). PSC-IBD (PSC-UC and PSC-CD) patients with OLT had severe disease in the left colon and rectum ($P = 0.04$).

CONCLUSION

PSC-UC represents a distinct form of IBD. The different disease phenotype in PSC-IBD patients with OLT may support liver-gut axis interaction, however warrants clinical attention and further research.

Key words: Primary sclerosing cholangitis; Inflammatory bowel disease; Liver transplantation; Inflammation; Pathologic features; Clinical associations

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Core tip: This is a retrospective study evaluating biopsies and clinical features of primary sclerosing cholangitis (PSC) patients with and without inflammatory bowel disease (IBD) in comparison to subjects with ulcerative colitis (UC) or Crohn disease (CD). Patients with PSC-UC had a different disease distribution characterized by right sided colitis, a milder disease course with lower activity scores in biopsies, less need for colectomy, and less steroids compared to UC. PSC-CD patients were rare but had more ileal inflammation compared to PSC-UC. PSC-No IBD patients showed similar characteristics to PSC patients in general and only one patient received orthotopic liver transplantation (OLT) in this group. Ten PSC-UC patients received OLT in contrast to no patients with PSC-CD. The need for OLT in PSC-IBD (PSC-UC and PSC-CD) correlated with rectal involvement and higher activity scores in the left colon biopsies in comparison to patients without OLT. This may require clinical attention since the both the intestinal and liver disease seem to be “severe” in this group further supporting the importance of gut-liver interaction in these patients.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a progressive chronic cholestatic disease of the intrahepatic and extrahepatic biliary tree. Most patients will require orthotopic liver transplant (OLT) within 8 years^[1]. PSC is associated with inflammatory bowel disease (IBD) in 70%-80% of patients^[1]. Although ulcerative colitis (UC) is most often associated with PSC^[2,3], a minority of patients with PSC and IBD will have Crohn disease (CD)^[3,4]. The histopathologic and endoscopic features of IBD in patients with PSC-IBD have been reported to differ from patients with IBD only. There is generally less severe colitis and more pancolitis, or right-sided disease, in patients with PSC-UC compared to those with UC only^[5-7]. Some studies have also suggested that there is increased rectal sparing and ileitis in PSC-UC^[1], however, others have not confirmed these findings^[5,6]. The histopathology of PSC-CD is not well characterized, though patients with PSC-CD are reported to have more colitis and less ileitis compared to those with CD without PSC^[6,8]. Further, increased numbers of lamina propria IgG4 positive plasma cells in active and inactive PSC-UC compared to active and inactive CD and inactive UC have been reported suggesting immunophenotypic differences^[9].

PSC-IBD may also have a distinct pathogenesis. Genetic studies have shown that HLA class II alleles associated with UC are different from those found in patients with PSC and patients with PSC-UC^[10]. Further, known IBD-associated polymorphisms such as *TLR-4*, *CARD15*, *CARD4*, *SLC22A4*, *SLC22A5*, *DLG5*, and *MDR1* are not found

at increased frequency in patients with PSC, PSC-UC, or PSC-CD^[10]. In addition patients with PSC and PSC-IBD have a similar gut microbial profile, which is different from patients with UC only and healthy controls, suggesting a microbiome connection^[11,12].

There is also evidence that gut-liver cross talk may contribute to the pathogenesis of PSC-IBD both in human studies and animal models^[13,14]. Hepatic disease severity in PSC is inversely associated with colitis in PSC-UC patients. PSC-UC patients who require OLT have been reported to have less severe colitis than those patients who do not^[13-15]. Studies have also shown that severe IBD pre and post OLT is associated with recurrent disease in the allograft^[18-20]. In patients with PSC-IBD, aberrant expression of gut-specific molecules in liver and liver-restricted molecules in gut likely provide a mechanism for T and B cell trafficking between the gut and the liver, and suggest the presence of several mechanisms for liver-gut cross talk^[13].

Based on observed differences in distribution of colonic involvement, severity of inflammation, rate of colorectal neoplasia and clinical outcomes, PSC-IBD represents a unique form of IBD, distinct from UC or CD without PSC^[6,8,21].

In light of the current literature findings, we studied PSC patients with and without IBD, who were referred to our center. Our study had two aims: (1) To describe the histopathologic features of PSC-UC and PSC-CD in comparison to UC and CD in the absence of PSC; and (2) To study the associations between clinical and histopathologic features of PSC-IBD with OLT.

MATERIALS AND METHODS

Study population

This study was approved by the institutional review board. This study was exempt from patient consent since the data was analyzed in an anonymous fashion. The archives of the Lauren V. Ackerman Laboratory at Barnes-Jewish Hospital were searched for ileal and colonic biopsies and resections from patients with UC only, CD only, PSC-UC, PSC-CD, as well as patients with PSC without IBD. Colonic and ileal biopsies and resection specimens from well-characterized patients 18 years old and older between 1999 and 2013 were identified. In all cases of IBD, the diagnosis was confirmed by the treating gastroenterologist following established guidelines^[22]. In all cases of PSC, the diagnosis was based on the presence of characteristic clinical and radiographic findings. In cases of PSC with normal colonic biopsies, the medical record was reviewed for subsequent development of inflammatory bowel disease. If IBD was later diagnosed, the cases were excluded. PSC-UC and UC patients, and PSC-CD and CD patients, were matched for age and IBD duration. Clinical data including gender, age, age at IBD diagnosis, age at PSC diagnosis, IBD duration, medical treatment, colectomy, OLT, development of dysplasia, and follow-up duration were collected from the electronic medical record. In addition, colonoscopic findings in PSC-UC and PSC-CD patients were also recorded.

Histologic analysis

For each patient with IBD, all available biopsy reports were screened, the most inflamed biopsy set with sufficient tissue, available in our files was selected for histologic and immunohistochemistry analysis. For patients with PSC-no IBD, the earliest biopsy was selected. Of note, the site of biopsies was obtained from endoscopy reports as designated by the submitting gastroenterologist.

The hematoxylin and eosin stained slides generated for routine clinical diagnosis were reviewed by two of the authors (IN and JAC). Histologic review was blinded to the clinical diagnosis, and disagreement as to interpretation of histologic features was resolved by consensus.

For each case the following features were noted at sites that were sampled: Distribution and presence of active inflammation, architectural distortion, basal plasmacytosis, Paneth cell metaplasia (left colon and rectum), and pyloric gland metaplasia. Pancolitis was defined as both right and left colonic disease, irrespective of rectal involvement. The presence of ileitis and granulomas was also noted. The degree (severity) of active inflammation at each site was also graded semi-quantitatively, and an activity score was assigned to each biopsy as described by Joo *et al*^[5]. A score of 0 was assigned if there was no active inflammation, (1) If less than 50% of crypts showed neutrophilic cryptitis and/or crypt abscesses; (2) If greater than 50% showed neutrophilic cryptitis and/or crypt abscesses; and (3) If there was surface erosion or ulceration^[5] (Figure 1).

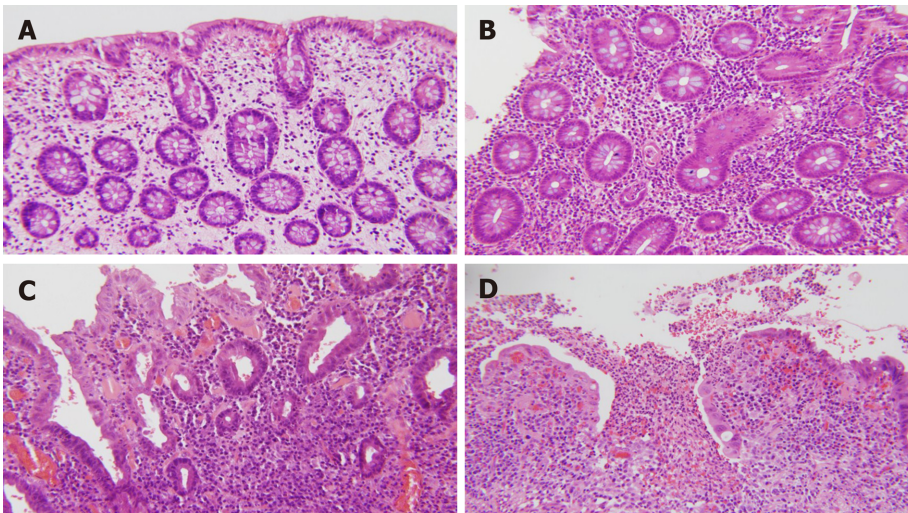


Figure 1 The degree of active inflammation was graded on a 4 tier scale (0-3). A: Cases without active inflammation were scored as 0 (200 \times); B: Cases with cryptitis and/or crypt abscesses involving less than 50% of the crypts were scored as 1 (200 \times); C: Cases with cryptitis and/or crypt abscesses involving more than 50% of the crypts were scored as 2 (200 \times); D: Ulceration was scored as 3 (200 \times).

Statistics

The statistical methods for this work include summary descriptive statistics for continuous outcomes and categorical outcomes and were performed by a certified biostatistician (YY). For continuous outcomes, the descriptive statistics include minimum, maximum, mean, standard deviation, and median. For categorical outcomes, the proportions in each category are produced. These descriptive statistics are produced for entire patient sample and for subgroups of patients. Two-sample *t* test or Wilcoxon test is used to compare a continuous outcome between two groups and AVONA is used to compare a continuous outcome among three or more groups. For a binary outcome, we used Chi-square (χ^2) test or Fisher's exact test to compare the proportion between two groups, and logistic regression model to compare the proportions among three or more groups. The *P* values are not adjusted for multiple comparisons since the study is exploratory in nature.

In addition, multivariable logistic regression is used to explore the relationship among liver transplantation (outcome variables) and a set of variables (independent variables) including disease distribution and severity, colectomy, and medical treatment in UC, CD, PSC-UC, and PSC-CD groups. Forward step-wise selection procedure was used for variable selection. The entry level of forward selection was set at 0.2 and 0.05 to compare the results. Calculations were performed using SAS® 9.4 software. *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Study population, clinical and histologic features

PSC-UC patients: Twenty-six PSC-UC patients and 28 age and IBD disease duration matched UC controls were identified (Table 1). Seventeen (65%) PSC-UC patients were male and had an average age of 37 years. The average age at IBD diagnosis was 27 years, and the average age at PSC diagnosis was 32 years. UC preceded PSC in 12 patients (46%), PSC preceded UC in 8 patients (31%), and in 5 patients (19%) the diagnoses were reached simultaneously. For one patient, the temporal relationship was not known (4%). The average duration of IBD was 10.8 years. Ten (38%) patients required OLT. Of these, UC preceded PSC in 6 patients, PSC preceded UC in 2 patients, and 2 patients had the diagnoses made concurrently. The average duration of follow-up was 8.2 years; and only 4 patients (15%) developed low grade dysplasia during this time. No high-grade dysplasia or cancer were noted in PSC-UC group, whereas 1 patient developed cancer in UC controls and 4 patients were noted to have low grade dysplasia. Except one patient who died of sepsis, all PSC-UC patients were alive at the time of data collection.

Between the PSC-UC and the UC group, there was no significant difference in gender (*P* = 0.9), age at IBD diagnosis (*P* = 0.3), duration of IBD (*P* = 0.5), duration of

Table 1 Clinical characteristics of Primary sclerosing cholangitis-Ulcerative colitis and Primary sclerosing cholangitis-Crohn disease patients (in all available patients, treatment data not mutually exclusive), *n* (%)

	PSC-UC	UC	<i>P</i> value	PSC-CD	CD	<i>P</i> value	<i>P</i> value (PSC-UC vs PSC-CD)
Male	17 (65)	18 (64)	0.9	5 (83)	2 (22)	0.03 ^a	0.4
Age at the time of biopsy (yr) (mean ± SD)	37 ± 14.61	41 ± 15.55	0.4	38 ± 14.93	32 ± 10.21	0.2	0.8
Age at IBD diagnosis (yr) (mean ± SD)	27 ± 14.38	30 ± 10.61	0.3	33 ± 14.77	22 ± 10.48	0.09	0.2
Age at PSC diagnosis (yr) (mean ± SD)	32 ± 13.96	NA	NA	31 ± 12.87	NA	NA	0.8
Interval between PSC and IBD diagnoses (yr) (mean ± SD)	9 ± 10.45	NA	NA	1.4 ± 1.6733	NA	NA	0.1
IBD duration at biopsy (yr) (mean ± SD)	10.8 ± 10.84	9 ± 9.19	0.5	7.00 ± 5.18	9.8 ± 4.06	0.5	0.3
PSC duration (yr) (mean)	6	NA	NA	12.33	NA	NA	0.8
Medical treatment							
Ursodiol	9 (35)	0	0.0006 ^a	2 (33)	0	0.1	1
Steroids	8 (31)	19 (68)	0.01 ^a	2 (33)	2 (22)	1	1
ASA	15 (58)	17 (61)	1	2 (33)	6 (67)	0.3	0.4
Anti-TNF	5 (19)	7 (25)	0.7	1 (17)	7 (78)	0.04 ^a	1
Immunomodulators	4 (15)	8 (29)	0.3	2 (33)	3 (33)	1	0.3
Colectomy	10 (38)	19 (68)	0.03 ^a	0	1 (11)	0.9	0.9
OLT	10 (38)	NA	NA	0	NA	NA	0.1
Follow-up duration (yr) (mean ± SD)	8.2 ± 4.61	8.2 ± 5.75	0.9	4.8 ± 2.23	5.9 ± 3.14	0.7	0.1
Development of dysplasia/cancer	4 (15)	5 (18)	0.8	0	0	1	0.9
Resection used in histologic and IHC analysis	6 (23)	9 (32)	0.4	0	3 (33)	0.9	0.9

^a*P* < 0.05. PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; CD: Crohn disease; SD: Standard deviation; ASA: 5-aminosalicylic acid; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease; OLT: Orthotopic liver transplantation; IHC: Immunohistochemistry; NA: Not available.

follow-up (*P* = 0.9), or development of dysplasia (*P* = 0.8). PSC-UC patients were less frequently treated with steroids (31%) compared to 68% of UC patients (*P* = 0.01), and patients with PSC-UC (38%) underwent colectomy less frequently than UC patients (68%) (*P* = 0.03).

Colonoscopy reports (not shown in tables) were available in 21 PSC-UC patients (1 flexible sigmoidoscopy). The five remaining patients, who did not have colonoscopy, had colectomy and the gross findings were noted. The colonoscopy was normal in three patients. Terminal ileum was noted to be normal in 57% of the cases (15 patients), whereas it was noted to be involved in 7% (2 patients), and data was not available in the rest. Pancolitis was the most commonly noted finding in 65% of all cases (17 patients). Isolated right-sided involvement was seen in only one patient (3%), and right side predominant disease was seen in four (15%). Rectal involvement and sparing were noted in four patients each (15%). Colonoscopic and gross examination correlated with the histologic findings in 50% of the patients.

A total of 181 biopsies and 15 resections from all patients were examined (Table 2). The PSC-UC group had more patients with disease confined to only the right side of colon compared to the UC group (29% vs 4%, *P* = 0.03). In contrast, isolated left sided disease was more commonly noted in UC patients (26% vs 4%, *P* = 0.06). Rectal involvement between the two groups was similar. No significant difference in pancolitis was observed between the two groups (67% vs 63%, *P* = 0.8).

The average activity score in right colon was higher in the PSC-UC group compared to UC (1.25 vs 0.96), however this did not reach statistical significance (*P* = 0.2). Left colon and rectum were less inflamed (lower activity scores) in the PSC-UC group (*P* = 0.03 and *P* = 0.0006). PSC-UC patients also had less active inflammation as an average of all sites examined compared to the UC group (*P* = 0.0005). Further, the PSC-UC patients showed less basal plasmacytosis in the left colon and rectum compared to the

Table 2 Histopathologic features of Primary sclerosing cholangitis-Ulcerative colitis and Primary sclerosing cholangitis-Crohn disease patients, *n* (%)

	PSC-UC ¹	UC ¹	<i>P</i> value	PSC-CD ¹	CD ¹	<i>P</i> value	<i>P</i> value (PSC-UC vs PSC-CD)
Distribution of disease							
Right colon only	7 (29)	1 (4)	0.03 ^a	3 (50)	2 (33)	0.6	0.3
Left colon only	1 (4)	7 (26)	0.06	0	0	1	0.9
Left and right colon	16 (67)	17 (63)	0.8	3 (50)	4 (67)	0.6	0.4
Rectum	15 (68)	18 (90)	0.1	1 (20)	5 (83)	0.055	0.07
Ileitis	4 (31)	1 (7)	0.1	5 (100)	4 (67)	0.9	0.9
Severity of disease (active inflammation)							
Right colon (average activity score)	1.25	0.96	0.2	1.5	1	0.3	0.5
Left colon (average activity score)	1.2	1.82	0.03 ^a	0.83	0.86	0.9	0.4
Rectum (average activity score)	0.96	2.05	0.0006 ^a	0.2	1.17	0.1	0.1
Ileum (average activity score)	0.31	0.07	0.2	1	1	1	0.01 ^a
Max average activity score at any site	1.46	2.29	0.0005 ^a	1.83	1.67	0.7	0.3
Architectural distortion							
Right colon	24 (100)	21 (78)	0.9	6 (100)	6 (100)	1	1
Left colon	24 (96)	26 (93)	0.6	6 (100)	7 (100)	1	0.9
Rectum	18 (78)	19 (95)	0.1	5 (100)	5 (83)	0.9	0.9
Any site	26 (100)	28(100)	1	6 (100)	9 (100)	1	1
Paneth cell metaplasia							
Left colon	11 (44)	14 (50)	0.7	1 (17)	4 (57)	0.1	0.2
Rectum	5 (22)	6 (30)	0.5	1 (20)	0	0.9	0.9
Basal plasmacytosis							
Right colon	14 (58)	13 (48)	0.5	2 (33)	3 (50)	0.5	0.3
Left colon	11 (44)	22 (79)	0.01 ^a	0	4 (57)	0.9	0.9
Rectum	6 (26)	19 (95)	0.0004 ^a	1 (20)	3 (50)	0.3	0.7
Any site	16 (62)	27 (96)	0.01 ^a	3 (50)	7 (78)	0.2	0.6
Pyloric gland metaplasia							
Right colon	2 (8)	1 (4)	0.7	1 (17)	1 (17)	1	0.6
Left colon	0	0	1	0	0	1	1
Any site	2 (8)	1 (4)	0.5	1 (17)	1 (11)	0.7	0.5
Granulomas							
Any site	1 (4)	0	0.9	1 (17)	6 (67)	0.08	0.3

^a*P* < 0.05.¹In all biopsied sites. PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; CD: Crohn diseases; NA: Not available.

UC group (*P* = 0.01 and *P* = 0.0004, respectively), and similar frequencies of basal plasmacytosis in the right colon (*P* = 0.5). No difference was noted between the PSC-UC group and the UC group in distribution of architectural distortion, or Paneth cell or pyloric gland metaplasia.

PSC-CD patients: Six patients with PSC-CD and 10 age and IBD disease duration matched CD controls were identified (Table 1). Five of PSC-CD patients were male (83%) and they had an average age of 38 years, average age of IBD diagnosis of 33

years, and average age of PSC diagnosis of 31 years. The average duration of IBD was 7 years, and the average duration of follow-up was 4.8 years. None required OLT. In one patient the diagnosis of CD preceded PSC (17%), in two patients the diagnosis of PSC preceded CD (33%), in two patients the conditions were diagnosed simultaneously (33%), and in one patient the temporal relationship was unknown (17%). None of the patients within study or control groups developed dysplasia during follow-up. All PSC-CD patients were alive at the time of data collection.

The PSC-CD group had a male predominance compared to the CD group ($P = 0.03$). The PSC-CD and CD groups there were similar in terms of age at IBD diagnosis ($P = 0.09$), IBD duration ($P = 0.5$), need for colectomy ($P = 0.9$), duration of follow-up ($P = 0.7$), and development of dysplasia ($P = 1.0$). The CD controls received more anti-tumor necrosis factor (TNF) than the PSC-CD group (78% *vs* 17%, $P = 0.04$), but otherwise the treatment regimens were similar.

Endoscopic data was available (not shown in tables) in all PSC-CD patients. Inflammation or focal strictures within terminal ileum were noted in 50% of the patients. Isolated right-sided colitis was noted in one patient (16%), pancolitis in two (33%) and normal/inactive disease was noted in the remaining three (50%). Colonoscopy reports correlated with the histologic findings in four patients (66.6%).

A total of 68 biopsies and 3 resections were examined (Table 2). Ileal involvement was noted in all PSC-CD patients (sampled in five out of six) and in 67% of the CD group. Although PSC-CD patients had higher activity scores in right colon (1.5 *vs* 1), this did not reach statistical significance ($P = 0.3$). Rectum was mostly spared in PSC-CD (20% *vs* 83% involvement) compared to CD ($P = 0.055$). Six of the CD patients were noted to have granulomas, whereas this was noted in only one PSC-CD patient (67% *vs* 17%, $P = 0.08$). Other histologic parameters were similar between the two groups.

PSC-UC patients compared to PSC-CD: The PSC-UC and the PSC-CD groups showed similar characteristics in terms of gender, age at biopsy, age at IBD diagnosis, disease duration of IBD and PSC, type of medical treatment, follow-up duration, and development of dysplasia (Table 1 and 2). Further, there was no significant difference between the two groups in age at PSC diagnosis and interval between PSC and IBD diagnosis. It is noteworthy that no PSC-CD patients required OLT in comparison to 10 of 26 patients (38%) with PSC-UC, however this difference did not reach statistical significance ($P = 0.1$).

Histologically, ileal involvement was more common in PSC-CD compared to PSC-UC (100% *vs* 31%, $P = 0.9$), however this did not reach statistical significance. In addition, the activity score in terminal ileum in the PSC-CD group was higher compared to PSC-UC (1.0 *vs* 0.31, $P = 0.01$). PSC-UC patients had more rectal involvement compared to PSC-CD (68% *vs* 20%, $P = 0.07$), whereas right-sided colitis was more common in the PSC-CD group (50% *vs* 29%, $P = 0.3$). All other histologic characteristics were similar.

PSC-no IBD patients compared to PSC-UC and PSC-CD: Four patients with PSC-no IBD were identified (not shown in tables). One patient was male, and the average age was 53 years, which was older than those in the PSC-UC and PSC-CD groups, but the difference did not reach statistical significance ($P = 0.1$ and $P = 0.2$). The average age at PSC diagnosis in the PSC-no IBD group was 51.8 years, which was older compared PSC-UC or PSC-CD groups ($P = 0.053$ and $P = 0.09$). The average PSC duration was 1.25 years, which was shorter than the duration observed in the PSC-UC and PSC-CD groups, but the difference did not reach statistical significance ($P = 0.1$ and $P = 0.2$). The mean follow-up duration for the PSC-no IBD group was 7 years. Only one of the four patients in the PSC-no IBD group required OLT. This was not significantly different than the rate of transplantation observed in the PSC-UC or PSC-CD groups ($P = 0.6$ and $P = 0.9$). All PSC-no IBD biopsies were histologically normal. All PSC-no IBD patients were alive at the time of data collection (2016).

Patients with OLT: A total of 11 patients received OLT (Table 3). Nine were men (89%) and 2 were women (19%). Ten of these patients had PSC-UC, and one had PSC-no IBD. Of note, none of the patients with PSC-CD required OLT. However, compared to PSC-UC, the difference did not reach statistical significance ($P = 0.1$). The demographic, clinical, and treatment modalities of patients with OLT did not differ from patients who did not require OLT (not shown in tables).

Five of eleven biopsies (45%) were obtained in the post-transplant period. Of these, three patients did not have material prior to OLT, and one developed IBD following OLT. The single patient who had a biopsy prior to OLT showed similar disease distribution compared to the biopsy that was included in the study.

Table 3 Select clinical and histologic features of patients with orthotopic liver transplantation

Diagnosis	Gender	Age (yr)	Age at Dx of IBD (yr)	Age at Dx of PSC (yr)	Colitis duration (yr)	Colectomy	Time of sample	Distribution of IBD	Rectal involvement	Follow-up (yr)	Medical treatment for IBD
PSC UC	F	45	19	40	26	No	Post-OLT	Right and left	Not involved	8	Ursodiol
PSC UC	F	18	3	27	15	Yes	Pre-OLT	Right and left	Involved	15	Azathioprine
PSC UC	M	61	57	57	4	No	Pre-OLT	Right and left	Involved	10	Mesalamine
PSC UC	M	52	18	42	34	Yes	Pre-OLT	Right and left	Involved	16	Ursodiol, azathioprine
PSC UC	M	24	19	20	5	Yes	Pre-OLT	Right and left	Involved	7	Steroids, mesalamine
PSC UC	M	19	18	15	1	No	Post-OLT	Right and left	Involved	13	Mesalamine
PSC UC	M	22	20	20	2	Yes	Post-OLT	Right and left	Involved	4	Mesalamine, azathioprine, infliximab, steroids
PSC UC	M	55	52	40	3	No	Post-OLT	Right only	Involved	17	Mesalamine
PSC UC	M	60	34	58	26	No	Pre-OLT	Left only	Involved	4	Mesalamine
PSC UC	M	49	29	35	20	Yes	Post-OLT	Right and left	Not involved	10	Steroids
PSC NIBD	M	57	NA	57	NA	NA	Pre-OLT	NA	NA	14	NA

NIBD: No inflammatory bowel disease; F: Female; M: Male; Dx: Diagnosis; NA: Not available; PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; CD: Crohn disease; IBD: Inflammatory bowel disease; OLT: Orthotopic liver transplantation; NA: Not available.

Six biopsies were obtained pre-OLT. Four patients did not have material available for examination in the post-OLT period (Notably, one of these four patients had a recent biopsy, which showed normal colon throughout). Of the remaining two, one showed similar disease distribution, and the other one showed isolated right colon involvement (the biopsy included for analysis shows rectal involvement).

PSC-UC patients with OLT had more severe inflammation (higher activity scores) in the left colon compared with patients not requiring OLT (mean activity score of 1.7 *vs* 0.86) and a trend was observed ($P = 0.06$, not shown on tables).

Since none of the PSC-CD patients required OLT, analysis of histologic and clinical features in relation to OLT was not possible. Isolated right-sided disease and involvement of both right and left colon was noted in 3 patients each, whereas rectal involvement was seen in only one.

When PSC-UC and PSC-CD were combined as PSC-IBD, patients with PSC-IBD who had OLT showed a different phenotype characterized by having more rectal involvement ($P = 0.04$, Table 4). In addition, left colon and rectum were more inflamed ($P = 0.04$ for each respectively, Table 4). Multivariate analysis also confirmed that patients with OLT had severe colitis in their left colon ($P = 0.04$).

DISCUSSION

The clinical characteristics of PSC-UC compared to UC have been the topic of a number of studies. The majority of these describe this entity predominantly based on endoscopic, and clinical findings with little to no details of pathologic features^[8,23]. However only a handful of investigators report some histologic features^[5,15]. In our opinion, the first and most detailed histologic description of PSC-UC is provided by Joo *et al*^[5]. Our group further extends some of their observations not only to PSC-UC patients but also to PSC-CD and PSC-no IBD groups with histologic characterization and studied their correlation with clinical outcomes and OLT.

Although considered as a different phenotype of IBD, some controversy remains as to the precise characterization of the histologic and endoscopic features of PSC-UC. Some authors have observed increased pancolitis in PSC-UC compared to UC^[5,6],

Table 4 Select histologic and clinical features of Primary sclerosing cholangitis-Inflammatory bowel disease patients with and without orthotopic liver transplantation, *n* (%)

	PSC-IBD with OLT ¹	PSC-IBD no OLT ¹	<i>P</i> value (PSC-IBD with OLT vs PSC-IBD no OLT)
Distribution			
Right colon only	1 (10)	9 (30)	0.1
Left colon only	1 (19)	0 (0)	0.3
Right and left colon	8 (80)	11 (37)	0.2
Rectum	8 (80)	8 (44)	0.04 ^a
Ileum	2 (33)	7 (58)	0.6
Dysplasia	3 (30)	1 (4.7)	0.08
Treatment			
Ursodiol	2 (20)	9 (45)	0.2
Steroids	3 (30)	7 (33)	1
ASA	6 (60)	11 (52)	1
Anti-TNF	1 (10)	5 (24)	0.6
Immunomodulators	3 (30)	3 (14)	0.3
Colectomy	5 (50)	5 (22)	0.2
Severity of acute inflammation (average activity score)			
Right colon	1.5	1.2	0.4
Left colon	1.7	0.86	0.04 ^a
Rectum	1.3	0.6	0.04 ^b
Ileum	0.3	0.6	0.3
IBD duration (yr) (mean ± SD)	13.6 ± 12	8.5 ± 8.8	0.3

¹In all available patients and biopsied sites.^aUni and multivariate analysis (*P* < 0.05).^bUni and multivariate analysis (*P* < 0.05). SD: Standard deviation; ASA: 5-aminosalicylic acid; Anti-TNF: Anti-tumor necrosis factor; IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis; OLT: Orthotopic liver transplantation.

whereas others have noted an increased right-sided distribution in PSC-UC patients^[7]. Loftus *et al*^[8] found significantly more pancolitis in the PSC-IBD group compared to UC. However, their study group is heterogeneous and included both PSC-CD and PSC patients with indeterminate colitis. In addition, distribution of disease was determined by review of endoscopic and/or pathology reports, but a secondary review of the pathology material was not performed. Isolated right-sided disease (1 patient) or right colon predominant disease (4 patients) was noted in a small percentage of our PSC-UC patients by endoscopy. However, histologic examination showed that PSC-UC patients had more isolated right-sided disease (29% *vs* 4%, *P* = 0.03) compared to UC control patients further reiterating the value of pathologic examination. Ileal involvement was also more commonly noted in PSC-UC (31% *vs* 7%), although not statistically significant. No significant difference in pancolitis was observed (67% *vs* 63%) between the two groups.

The severity of disease also appears to be different in PSC-UC. Most literature reports a more severe disease in right colon compared to left and less severe disease overall^[5,7,15]. Backwash ileitis and rectal sparing in this group of patients were also more commonly reported compared to UC patients without PSC^[15,8].

Similar to the literature, PSC-UC patients in our cohort also had more inflammatory activity in right colon compared to UC patients (average activity score of 1.25 *vs* 0.96), although this did not reach statistical significance. PSC-UC patients also had milder colitis of the left colon (*P* = 0.03) and rectum (*P* = 0.0006), and a lower activity score overall (*P* = 0.0005) compared to UC patients, confirming the previously reported findings. Ileal inflammation was also more severe in PSC-UC but this did not reach

statistical significance. In addition, basal plasmacytosis was less frequently noted in the left colon and rectum of PSC-UC patients compared to UC patients ($P = 0.01$ and $P = 0.0004$), suggesting less chronic damage in the distal colon and rectum in the former.

Clinically, PSC-UC also manifests itself as a “milder form of colitis” compared to UC with no PSC, requiring less steroid treatments, fewer hospitalization and even fewer colectomies^[23,24]. Our results are also in keeping with this. In our study, PSC-UC patients required fewer courses of steroids (31% *vs* 68%, $P = 0.01$) and fewer colectomies (38% *vs* 68%, $P = 0.03$) compared to, age- and IBD duration-matched, patients with UC only. It has been noted that patients with PSC-UC have higher rates of dysplasia and colorectal malignancy compared to patients with UC only^[25,26]. In our cohort, however, similar rates of dysplasia/cancer were observed in PSC-UC patients and UC patients (15% compared to 18%, $P = 0.8$).

Though PSC is much more frequently associated with UC, a minority of PSC patients will have CD. The number of patients with PSC-CD is very limited and histopathologic features are poorly characterized. To best of our knowledge, our study is the first to evaluate this difference on the basis of detailed microscopic evaluation. None of the PSC-CD patients had classic histologic features of CD and, they showed some differences compared to CD controls. First, a male predominance was noted (83% *vs* 22%, $P = 0.03$). Histologically involvement of ileum was more common (100% *vs* 67%, $P = 0.9$), and more cases had isolated right colon disease (50% *vs* 33%, $P = 0.3$). Rectal disease on the other hand was more common in the CD group (83% *vs* 20%, $P = 0.055$), which was also more inflamed (1.17 *vs* 0.2, $P = 0.1$). Granulomas were less frequently identified in PSC-CD compared to CD (17% *vs* 67%, $P = 0.08$). Although, terminal ileum strictures were noted in two PSC-CD patients, isolated small intestine disease was not noted in any of them. Clinically, CD patients received more anti-TNF compared to PSC-CD (78% *vs* 17%, $P = 0.04$). Loftus *et al*^[8] describe similar features in their 5 PSC-CD patients and report that only 2 of these had diagnostic features of CD. In a Norwegian study, none of the 15 PSC-CD subjects were noted to have characteristic features of CD; in addition some of these were later classified as PSC-UC^[15]. None of our PSC-CD subjects has been re-classified to PSC-UC at the time of data collection, and in general they have milder disease than CD patients.

Differences in disease distribution and severity were also noted between PSC-CD and PSC-UC. PSC-CD patients had more right sided (50% *vs* 29%, $P = 0.3$) and ileal disease (100% *vs* 31%, $P = 0.9$) compared to PSC-UC, whereas rectal involvement was more common in PSC-UC (68% *vs* 20%, $P = 0.07$). When compared to PSC-UC patients, the severity of inflammation in ileum (1 *vs* 0.31) was more profound in PSC-CD. PSC-CD did not significantly differ from PSC-UC in terms of patient characteristics, treatment modalities and clinical outcomes. It is likely that only the two patients with ileal strictures indeed represent PSC-CD in our cohort and the rest simply represents PSC-UC. However, the presence and severity of ileal disease compared to PSC-UC patients is compelling. Of note, distinction between PSC-CD *vs* PSC-UC was done by the treating gastroenterologist and it's certainly possible that these patients had other clinical findings that were not explicitly discussed in the patient's medical record, which led to the diagnosis. According to Jorgensen *et al*^[15], “True PSC-CD” is a very rare entity and all cases of PSC-UC and PSC-CD should be classified as PSC-IBD. Such a conclusion however, will require detailed histologic analysis similar to our study but in a larger patient population.

In addition to the PSC-CD patients, we also describe the clinical features of a small but unique group of patients with PSC but no IBD for the first time in the literature. These four patients had a mean follow-up of 7 years and showed similar clinical characteristics to PSC-UC and PSC-CD. Although this is a very small group of patients, and they still have the potential to develop IBD later, further study of these patients may provide insights into mechanisms for developing IBD. Whether this can be explained based on the microbiome^[11], liver-gut cross talk, undetected differences in inflammatory cell subtypes, genetic tendencies or a combination of these remains to be studied.

It has been noted that there is an increase in IgG4 positive plasma cells in patients with active and inactive PSC-UC compared to inactive UC, and active and inactive CD^[9] in rectal biopsies, suggesting that IgG4 positive cells may play a role in pathogenesis. We investigated this in our study population and stained the most inflamed biopsy with CD3, CD20, and IgG4. Although PSC-UC patients had higher numbers of IgG4 positive plasma cells, no difference in IgG4 cell counts were observed between PSC-UC and UC, PSC-CD and CD or PSC-UC and PSC-CD groups (not shown on tables). It should be noted that increased numbers of IgG4 positive plasma cells could be observed in a variety of inflammatory conditions^[27]. Typically, IgG4 related disorders display not only a lymphoplasmacytic infiltrate with increased

numbers of tissue IgG4 plasma cells (or increased IgG4: IgG ratio), but also with accompanying storiform fibrosis, and obliterative phlebitis^[28]. Therefore, in the absence of any of these findings, it is hard to make an argument that either PSC-UC or PSC-CD is an IgG4 driven processes.

It has been reported that the severity of IBD in patients with PSC-IBD can be related to the severity of the PSC. Marelli *et al*^[17] found that PSC-UC patients that required OLT had a milder clinical course and less inflammation on histology compared to PSC-UC not requiring OLT. Similar observations have been noted by others^[15,16]. However, others report that the severity of IBD in PSC correlates with liver disease both in humans and animal models, and patients with severe IBD tend to get recurrent PSC in the allograft^[14,19,20]. In our PSC-UC cohort, the severity of colitis in left colon showed a trend with OLT ($P = 0.06$, not shown on tables). When PSC-UC and PSC-CD groups are combined as PSC-IBD, patients with OLT had a disease phenotype characterized by rectal involvement ($P = 0.04$) and higher left colonic and rectal activity scores ($P = 0.04$ for each respectively). Severe inflammation of the left colon was also noted to be statistically significant in OLT patients in multivariate analysis ($P = 0.04$). It is certainly possible that changes in treatment modalities following transplantation have played a role and caused recurrent disease^[29] but this was not statistically significant in our cohort. Our results may be hindered by the heterogenous nature of their timeline (pre *vs* post-OLT), but we tried to overcome such biases by selecting the most inflamed set of samples, which likely represents the disease phenotype of patient's IBD. Nevertheless, severe inflammation of the left colon and rectum in PSC-IBD patients with OLT appears to be different than the “usual” pattern of disease in PSC-IBD, which is predominantly a right sided disease that manifests with milder inflammation in the left colon. Previous studies have supported the idea that the inflammatory state of the gut and the liver depend on each other. Although our statistical power is limited by our small cohort, based on our findings and other reports, it is not completely unreasonable to hypothesize that severe left sided colitis in our patients correlated with the need for OLT and alterations in liver-gut crosstalk may very likely have contributed to this potential phenotypic change^[13,19,20,30]. Unfortunately, data for disease recurrence is not available in our cohort, therefore it's unclear if severe left sided colitis also leads to recurrent PSC in the allograft.

In summary, our study confirms that PSC-UC is a distinct type of IBD based on the clinical and histologic findings in keeping with previous observations in the literature. PSC-CD, a cohort that has not been extensively studied before, showed more ileal and right colon involvement and rectal sparing compared to PSC-UC, but similar clinical features in general. These further supports the idea that PSC-CD and PSC-UC are part of a distinct PSC-IBD phenotype, but warrants further exploration. Similarly, PSC-no IBD patients may represent a distinct entity, and additional analysis of this unique but limited group of patients may provide insights into mechanisms and triggers for development of IBD in patients with PSC. PSC-IBD patients with OLT appear to have more frequent involvement of rectum and the severity of left sided colitis appears to be worse compared to patients who didn't receive OLT. This is an interesting disease phenotype that likely deserves clinical attention.

Our study has some limitations including sample size, heterogeneity of the specimens and potential referral bias of the patients. Despite the fact that we are a major referral center and we collected data from over a 10-year period, the case number is limited by the rarity of this entity. The strengths of the study include detailed clinical, histologic characterization and description of distinct groups including PSC-no IBD and PSC-CD. In addition, histologic and clinical findings of patients with OLT are described. Our study makes interesting observations in these groups, which have not been reported before. Since the ultimate patient outcomes are related to the liver disease, it is important to identify the disease characteristics of IBD in PSC patients that correlate with the need for OLT regardless of the IBD type. We hope our observations can be extended to larger cohorts, which may ultimately change our understanding of this disease and also how we treat our patients.

ARTICLE HIGHLIGHTS

Research background

Primary Sclerosing cholangitis (PSC) associated inflammatory bowel disease (IBD) is a unique form of IBD seen in patients with PSC. It has been characterized as to have right sided predominance in colon with less severe active inflammation. Most cases of PSC-IBD are classified as ulcerative colitis (UC), whereas rare cases exhibit features of

Crohn disease. There is thought to be a link between the IBD severity and the progression of PSC.

Research motivation

The need for better characterization of the pathologic findings in PSC-IBD patients and its association with liver transplantation may further support the “gut-liver axis” theory and has potential clinical implications. In addition, little is known regarding PSC-Crohn disease and its clinical outcomes.

Research objectives

The primary aims in this study were to characterize the colon and ileal findings in PSC patients at a tertiary care center, better define the histologic features of PSC-IBD, and explore if there is any correlation between the intestinal disease and liver transplant status, since this can impact patient management.

Research methods

This retrospective study was conducted in a single tertiary care center. Based on data search, cases with PSC and lower gastrointestinal biopsies were identified. Care was taken to examine the most inflamed biopsy. The hematoxylin and eosin slides were re-reviewed and several morphologic features were recorded. Pertinent clinical data was collected.

Research results

Our study confirmed the previously reported histologic findings in PSC-UC patients including a predominantly right sided involvement with overall less severity. We also described detailed histologic features of PSC-Crohn disease (CD) patients. None of the PSC-CD patients required liver transplantation, in contrast to ten PSC-UC patients. In our study, there was no correlation between the clinical parameters or treatment and orthotopic liver transplantation (OLT). When all PSC-IBD patients were analyzed together, severe left sided colitis correlated with the need for OLT.

Research conclusions

In our cohort, PSC-IBD patients with severe left sided and rectal disease required OLT more commonly than other PSC-IBD patients. This is rather interesting, since it may indicate that these patients are at increased risk for progression of their liver disease and this has not been reported before.

Research perspectives

The findings in this study further support the notion that gut and liver interact through several different mechanisms. Our results raise the possibility that an only a subset of patients with PSC-IBD (severe disease activity in left colon in our cohort) may be at increased risk for faster progression of liver disease, and eventually receive OLT. However, the contribution of other factors such as microbiome, genetic underpinnings, or others remain unanswered and should be further studied.

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Retrospective Study

Insulin receptor substrate 1 may play divergent roles in human colorectal cancer development and progression

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Abstract

BACKGROUND

Despite effective prevention and screening methods, the incidence and mortality rates associated with colorectal cancer (CRC) are still high. Insulin receptor substrate 1 (IRS-1), a signaling molecule involved in cell proliferation, survival and metabolic responses has been implicated in carcinogenic processes in various cellular and animal models. However, the role of IRS-1 in CRC biology and its value as a clinical CRC biomarker has not been well defined.

AIM

To evaluate if and how IRS-1 expression and its associations with the apoptotic and proliferation tumor markers, Bax, Bcl-xL and Ki-67 are related to clinicopathological features in human CRC.

METHODS

The expression of IRS-1, Bax, Bcl-xL and Ki-67 proteins was assessed in tissue samples obtained from 127 patients with primary CRC using immunohistochemical methods. The assays were performed using specific antibodies against IRS-1, Bax, Bcl-xL, Ki-67. The associations between the expression of IRS-1, Bax, Bcl-xL, Ki-67 were analyzed in relation to clinicopathological parameters, *i.e.*, patient age, sex, primary localization of tumor, histopathological type, grading, staging and lymph node spread. Correlations

Informed consent statement:

Patients were not required to give informed consent to the study because the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement:

All the Authors have no conflict of interest related to the manuscript.

Data sharing statement:

No additional data are available.

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between variables were examined by Spearman rank correlation test and Fisher exact test with a level of significance at $P < 0.05$.

RESULTS

Immunohistochemical analysis of 127 CRC tissue samples revealed weak cytoplasmatic staining for IRS-1 in 66 CRC sections and strong cytoplasmatic staining in 61 cases. IRS-1 expression at any level in primary CRC was associated with tumor grade (69% in moderately differentiated tumors, G2 *vs* 31% in poorly differentiated tumors, G3) and with histological type (81.9% in adenocarcinoma *vs* 18.1% in adenocarcinoma with mucosal component cases). Strong IRS-1 positivity was observed more frequently in adenocarcinoma cases (95.1%) and in moderately differentiated tumors (85.2%). We also found statistically significant correlations between expression of IRS-1 and both Bax and Bcl-xL in all CRC cases examined. The relationships between studied proteins were related to clinicopathological parameters of CRC. No significant correlation between the expression of IRS-1 and proliferation marker Ki-67, excluding early stage tumors, where the correlation was positive and on a high level ($P = 0.043$, $r = 0.723$).

CONCLUSION

This study suggests that IRS-1 is co-expressed with both pro- and antiapoptotic markers and all these proteins are more prevalent in more differentiated CRC than in poorly differentiated CRC.

Key words: Colorectal cancer; Insulin receptor substrate-1; Bax protein; Bcl-xL protein; Apoptosis; Antigen Ki-67

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Core tip: We analyzed the expressions of Insulin receptor substrate 1 (IRS-1), Bax, Bcl-xL and Ki-67 proteins in primary colorectal cancer (CRC). We found that IRS-1 expression was associated with tumor grade and histological type, and was more prevalent in more differentiated CRC. Interestingly, IRS-1 expression was significantly correlated with Bax and Bcl-xL, but not with Ki-67. We hypothesize that coexpression of IRS-1 and proapoptotic and antiapoptotic markers could result in a complex and diverse interplay characteristic for earlier stages in CRC.

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INTRODUCTION

Based on GLOBOCAN 2018 data, colorectal cancer (CRC) is the third most common cancer diagnosed across the world^[1]. Most of the relevant research is aimed at finding new prognostic factors or therapeutic strategy in order to reduce high CRC-related mortality. In addition to the numerous transcription factors and signaling molecules involved in the development of CRC, the role of insulin receptor substrate 1 (IRS-1) has been the subject of recent intense investigation^[2,3]. IRS-1 is a member of the IRS family (IRS-1 to IRS-6) and is generally considered to be a substrate of the insulin receptor (IR) and the insulin-like growth factor 1 (IGF-1) receptor (IGF-1R)^[4]. Activation of insulin signaling pathway is crucial in regulation of cell metabolism, while activation of IGF-1 signaling mediates processes, such as mitogenesis, differentiation and cell survival due to signal transmission in the phosphoinositol-3-kinase pathway and mitogen-activated protein kinase pathways^[5]. Signaling effectors that bind to IRS-1 include the phosphoinositol-3-kinase pathway, Grb-2, SHP-2, c-Crk, and NCK^[6-9]. Many of these signaling pathways have been implicated in carcinogenesis and cancer progression, thus IRS-1 has been proposed to play a central

role in determining the response of tumor cells to microenvironmental signals, including growth factors and hormones. Constitutive activation of IRS-1 has been found in various solid tumors^[10]. For instance, in ER-positive breast cancer cells, overexpression of IRS-1 enhances cell proliferation and reduces estrogen growth dependence^[11,12]. Also, overexpression of IRS-1 and IRS-2 in the mammary gland of mice was found to cause mammary tumorigenesis and metastasis^[13]. Intestinal epithelium express both the IR and the IGF-1R, and the levels of these receptors are higher in CRC compared with normal colonic mucosa^[14]. Accumulating evidence suggested that IRS-1 may be important component of the pathophysiologic mechanisms that underlie the colorectal carcinogenesis and tumor progression. Intestinal epithelial differentiation is regulated by multiple pathways, including β -catenin-dependent WNT signaling^[15]. Most CRC appear to initiate after inactivating mutations in the adenomatous polyposis coli gene, which results in uncontrolled cell proliferation through constitutive activation of WNT/ β -catenin signaling^[16]. In relevance, IRS-1 is highly upregulated in cells with exogenously-induced or constitutive β -catenin signaling and promotes transformation in cells that ectopically express β -catenin^[17]. Also, upregulation of IRS-1 by WNT/ β -catenin signaling in the mouse hepatocellular carcinoma model was found to play an important role in hepatocarcinogenesis^[18]. Furthermore, a study in the adenomatous polyposis coli min/+ mouse model showed that the intestinal tumorigenesis is attenuated by IRS-1 knock-out^[3]. Inhibition of growth of colon cancer cells was also observed as an effect of blocking the IGF-1R signaling by micro Ribonucleic Acid 145^[19].

Tumorigenesis is a multistep process, involving not only abnormal proliferation but also avoidance of apoptosis in transformed cells. The impairment of apoptosis is a critical step in tumor development but it also can contribute to therapeutic resistance as induction of apoptosis is a major cytotoxic mechanism of anticancer therapies^[20]. There are two primary apoptotic pathways: The extrinsic and the intrinsic, also called mitochondrial apoptotic pathway. In this work, we have focused on intrinsic apoptotic pathway and Bcl-2 family proteins that regulate programmed cell death. The Bcl-2 family consists of three subgroups of proteins. The prosurvival subfamily, which includes proteins such as Bcl-2 and Bcl-xL, protects cells from a wide range of cytotoxic insults, whereas two other subfamilies (Bax-like apoptotic subfamily and the BH3-only proteins) promote apoptosis. Tumor cells develop a variety of strategies to avoid apoptosis, including enhanced expression of antiapoptotic Bcl-2 family members, such as Bcl-xL and reduced expression of proapoptotic Bcl-2 family members, such as Bax^[21].

Numerous studies have shown that IRS-1 signaling contributes to tumor cell survival. In ER-positive breast cancer cells, suppression of IRS-1 expression accelerates apoptosis and renders cells more vulnerable to tamoxifen-induced cell death^[22]. Further, study in IRS-1-deficient mice have shown that reduced expression of IRS-1 increases apoptosis of crypt stem or progenitor cells and protects against intestinal tumors development^[3]. However, other evidence points to proapoptotic IGF-1R/IRS-1 functions^[23].

Here, we assessed IRS-1 expression in primary CRC and analyzed associations between the expression of IRS-1 and apoptotic (Bax, Bcl-xL) and proliferation (Ki-67) markers in relation to clinicopathological variables in CRC.

MATERIALS AND METHODS

Study design

To assess and compare immunohistochemical expression of IRS-1, Bax, Bcl-xL and Ki-67 tissue material obtained from 127 patients with pathologically confirmed diagnosis of CRC were analyzed. In order to obtain a sample reflecting the general population, the study was designed with wide range of inclusion criteria. The exclusion criteria included: (1) Age < 35 years; and (2) Known genetic predisposition to the development of colon cancer. We analyzed material from patients who underwent radical surgery with lymph node dissection.

Small biopsy specimens were excluded from the study. This study protocol was reviewed and approved by the Local Ethical Committee at the Medical University of Bialystok (Resolution No.: APK.002.105.2020).

Tissue samples

The postoperative material was fixed in 10% buffered formalin and paraffin-embedded. From paraffin blocks, 5 μ m sections were cut, deparaffinized, rehydrated

and stained with hematoxylin-eosin. Next, routine histopathological analysis of slides was performed in accordance with recommendation of World Health Organization.

Among 127 tumors, 57 had primary localization in rectum and 69 in colon. Histopathological analysis revealed 104 of adenocarcinoma cases and 23 of adenocarcinoma with mucosal component cases. Tumors were classified, according to their extramural depth of invasion, into two categories: pT1 + pT2 for tumors assessed as pT1 or pT2, which counted 11 cases and pT3 + pT4 for tumors assessed as pT3 or pT4, which counted 116 cases. Furthermore, in agreement with guidelines of World Health Organization, 69 tumors were classified as moderately differentiated (G2) and 87 tumors were classified as poorly differentiated (G3). Presence of metastases to regional lymph nodes was observed in 67 cases.

Immunohistochemical staining

Immunohistochemical assays were performed on formalin-fixed, paraffin-embedded tissue samples using primary antibodies against IRS-1, Bax, Bcl-xL, Ki-67 (Santa Cruz Biotechnology Incorporated, Santa Cruz, California, United States). To improve antigen expression, we applied pretreatment using Heat-induced Epitope Retrieval for 15 min in microwave. Next, the sections were incubated with blocking serum for 10 min to minimize false-positive staining. Tissues with confirmed immunohistochemical expression of IRS-1, Bax, Bcl-xL, Ki-67 served as positive control, while in negative control primary antibodies were substituted with phosphate-buffered saline. To visualize the target-antibody interaction, Dako Envision kit (Dako, Carpinteria, CA, United States) was used and 3,3'-Diaminobenzidine (DAB-kit, Dako Cytomation, Denmark) served as a chromogen.

Evaluation of biomarker expression was performed by two independent pathologists in 10 representative fields in each immunohistochemistry slide (magnification of 200 ×). The percentage of positive cells was scored as follows: 0, less than 10% immunoreactive cells in tumor; (1) 10%-50% immunoreactive malignant cells in tumor; and (2) More than 50% immunoreactive neoplastic cells in tumor.

Statistical analysis

The correlations between expression of IRS-1 and Bax, Bcl-xL, Ki-67 in primary CRC were analyzed in relation to clinicopathological parameters, including: patient age, sex, primary localization of tumor, histopathological type, grading, staging and lymph node spread. Correlations between variables were analyzed by Spearman rank correlation test with a level of significance at $P < 0.05$. Additionally, to estimate the strength of the correlation, Guilford's classification method was applied [correlation factor (r): 0.0-0.2 (slight); 0.2-0.4 (low); 0.4-0.7 (moderate); 0.7-0.9 (high); 0.9-1.0 (very high)]. Data analysis was conducted using STATISTICA PL v.12.0. software.

RESULTS

Expression of IRS-1 in CRC

Immunohistochemical analysis of CRC sections revealed weak cytoplasmic staining for IRS-1 in 66 CRC sections, while strong cytoplasmic staining for IRS-1 was observed in 61 cases of primary CRC. IRS-1 staining was not detected in negative controls. We observed that IRS-1 expression at any level in CRC was associated with moderately differentiated tumors (G2) (69% in G2 tumors compared with 31% in G3 tumors) and with histological type (81.9% in adenocarcinoma cases compared with 18.1% in adenocarcinoma with mucosal component cases) (Table 1).

Correlation of IRS-1 with Bax expression in CRC

The results demonstrated a moderate positive correlation between IRS-1 and Bax expression is statistically significant in all groups excluding cases of adenocarcinomas with mucosal component (mucinous adenocarcinoma), poorly differentiated tumors (G3) and early stage tumors (Table 2).

Correlation of IRS-1 with Bcl-xL expression in CRC

A positive correlation on a low to high level was found between IRS-1 and Bcl-xL in all cases, except patients characterized with tumor with primary localization in rectum, adenocarcinoma with mucosal component, poorly differentiated tumor (Table 2).

Table 1 Analysis of correlation between insulin receptor substrate 1 expression and clinicopathologic features, *n* (%)

Clinicopathological features		IRS-1 expression		<i>P</i> Fisher exact test
		Weak (0), <i>n</i> = 66	Strong (1+, 2+), <i>n</i> = 61	
Age (yr)	≤ 60 (<i>n</i> = 42)	22 (33.8)	20 (33.3)	0.552
	> 60 (<i>n</i> = 83)	43 (66.2)	40 (66.7)	
Gender	Male (<i>n</i> = 70)	37 (56.1)	33 (54.1)	
	Female (<i>n</i> = 56)	28 (42.4)	28 (45.9)	
Tumor Localization	Rectum (<i>n</i> = 57)	33 (50.8)	24 (39.3)	0.134
	Colon (<i>n</i> = 69)	32 (49.2)	37 (60.7)	
Histological Type ^a	A (<i>n</i> = 104)	46 (69.7)	58 (95.1)	< 0.001
	MA (<i>n</i> = 23)	20 (30.3)	3 (4.9)	
Histological differentiation ^a	G2 (<i>n</i> = 87)	35 (53.8)	52 (85.2)	< 0.001
	G3 (<i>n</i> = 39)	30 (46.2)	9 (14.8)	
Tumor size	pT1 + pT2 (<i>n</i> = 11)	7 (10.6)	4 (6.6)	0.312
	pT3 + pT4 (<i>n</i> = 116)	59 (89.4)	57 (93.4)	
Lymph node Involvement	Negative (<i>n</i> = 60)	29 (43.9)	31 (50.8)	0.438
	Positive (<i>n</i> = 67)	37 (56.1)	30 (49.2)	

Correlations were analyzed by Fisher exact test.

^a*P* < 0.05; *n*: Number of cases; *r*: Correlation coefficient; A: Adenocarcinoma; MA: Mucinous adenocarcinoma; G2: Moderately differentiated; G3: Poorly differentiated; N (-): Negative lymph node invasion; N (+): Positive lymph node invasion.

Correlation of IRS-1 with Ki-67 expression in CRC

No significant correlations were found between IRS-1 and Ki-67, excluding early stage tumors (pT1 + pT2) where the correlation was positive and on a high level (*P* = 0.043, *r* = 0.723) (Table 3). Interestingly, the same group of tumors was negative for coexpression of Bax and Bcl-xL.

Correlation of Bax with Bcl-xL expression in CRC

We also found statistically significant positive correlations between proapoptotic Bax and antiapoptotic Bcl-xL protein expression in all groups, except early stage tumors (pT1 + pT2) (Table 4).

DISCUSSION

The present study demonstrates positive correlations between IRS-1 expression and the presence of proapoptotic Bax as well as antiapoptotic Bcl-xL in primary CRC. These associations were prevalent in more differentiated CRC compared with less differentiated CRC. Previously, we found similar relationships in primary breast cancer (positive correlation between IRS-1 and Bax with *P* < 0.001 and *r* = 0.346 as well as IRS-1 and Bcl-xL with *P* < 0.001 and *r* = 0.315) and in lymph node metastases (positive correlation between IRS-1 and Bax with *P* = 0.037 and *r* = 0.356 as well as IRS-1 and Bcl-xL with *P* = 0.004 and *r* = 0.447)^[24]. Based on this data we assume that influence of IRS-1 on tumor cell survival mechanisms can be diverse and can be related to cancer stage.

In general, cell survival depends on the balance between expressed amounts of proapoptotic and antiapoptotic Bcl-2 family members. Increasing evidence suggested that function of Bcl-2 family proteins could be also regulated by its phosphorylation or dephosphorylation rates^[25]. Overexpression of IRS-1 was reported to suppress insulin-induced phosphorylation and activation of Bcl-2 protein^[26]. Another study documented that IRS-1-mediated signals lead to resistance to apoptosis induced by Transforming Growth Factor-β1 in hepatocellular carcinoma cells^[27]. Also, overexpression of IRS-1 in glioblastoma cells was found to promote cell survival^[28]. Moreover, a study on brown pre-adipocytes indicated that IGF-1 as well as insulin

Table 2 Analysis of correlations between Insulin receptor substrate 1, Bax and Bcl-xL expressions in primary colorectal cancer

Groups of patients		IRS-1-Bax			IRS-1-Bcl-xL		
		<i>n</i>	<i>P</i> value	<i>r</i>	<i>n</i>	<i>P</i> value	<i>r</i>
All		123	< 0.001	0.513 ^a	119	< 0.001	0.354 ^a
Age (yr)	≤ 60	40	< 0.001	0.661 ^a	41	0.001	0.481 ^a
	> 60	81	< 0.001	0.424 ^a	76	0.020	0.267 ^a
Sex	Male	67	< 0.001	0.497 ^a	63	0.011	0.319 ^a
	Female	55	< 0.001	0.537 ^a	55	0.002	0.415 ^a
Localization	Rectum	54	< 0.001	0.460 ^a	51	0.080	0.247 ^a
	Colon	68	< 0.001	0.536 ^a	67	< 0.001	0.446 ^a
HP-type	A	101	< 0.001	0.535 ^a	96	< 0.001	0.535 ^a
	MA	22	0.953	-0.013	23	0.841	-0.044
G	G2	84	< 0.001	0.523 ^a	83	0.006	0.300 ^a
	G3	38	0.263	0.186	35	0.474	0.125
T	pT1 + pT2	10	0.455	0.268	11	0.005	0.772 ^a
	pT3 + pT4	113	< 0.001	0.529 ^a	108	0.001	0.302 ^a
N	pN (-)	58	< 0.001	0.507 ^a	57	0.007	0.351 ^a
	pN (+)	65	< 0.001	0.493 ^a	62	0.007	0.336 ^a

Associations analyzed by Spearman's correlation rank test.

^a*P* < 0.05; *n*: Number of cases; *r*: Correlation coefficient; A: Adenocarcinoma; MA: Mucinous adenocarcinoma; G2: Moderately differentiated; G3: Poorly differentiated; N (-): Negative lymph node invasion; N (+): Positive lymph node invasion.

exerts antiapoptotic effect, which can be impaired by IRS-1 deletion and restored by its re-expression^[29]. It was also reported that overexpression of the IGF-1R in human CRC cell line (Hematocrit 116/IGF-1R) results in up-regulation of the antiapoptotic protein Bcl-xL^[30].

In contrast, several reports suggested that IRS-1 is either not critical or plays a negative role in cell survival. For example, in 32 D hematopoietic cells lacking IRS-1 expression, IGF-1R activation still protected cells from apoptosis, suggesting that non-IRS-1 pathways were involved in the process^[31]. Another study in the 32 D model system showed that IRS-1 expression sensitized cells to chemotherapy-induced death, but it did not affect the expression of pro- or antiapoptotic proteins^[32]. Furthermore, IRS-1 overexpression in transgenic mouse livers enhanced cell proliferation and caused up-regulation of *Fas* receptor but increased cell sensitivity to apoptosis^[33]. Another study demonstrated that IGF-1 treatment of MG63 osteosarcoma cells stimulated growth and proliferation but also mildly induced apoptosis through caspase-3 activation, annexin-V binding and deoxyribonucleic acid degradation^[23]. Surprisingly, the same study also showed coactivation of antiapoptotic signals such as Bad phosphorylation at serine 112. Thus, in this model, the increased growth induced by IGF-1 treatment might be balanced by activation of pro-death mechanisms. In line with this suggestion, our results show differential associations between expression of IRS-1 and Bax and Bcl-xL that depend on tumor size. IRS-1 and antiapoptotic Bcl-xL protein were positively correlated in cases of small tumors (pT1 + pT2) while in more extended tumors (pT3 + pT4) IRS-1 expression was positively correlated with Bcl-xL as well as proapoptotic Bax protein. Based on this, we assume that IRS-1 could play a fortifying role in fast growing tumors in which blood supply and nutrients become limited. Under these conditions, coactivation of antiapoptotic and proapoptotic pathways might provide balanced cell turnover and result in tumor progression. The precise mechanisms of how IRS-1 interplays with apoptotic proteins are not yet understood. The abovementioned study^[26] demonstrated that IRS-1 suppress apoptotic cell death induced by growth factor withdrawal probably through regulating phosphorylation of Bcl-2. The authors have also shown that IRS-1 was able to bind Bcl-xL but not Bax. Bcl-xL is capable of heterodimerize with Bax and counteracting its apoptotic effect. It is quite possible that during early step of tumor development, IRS-1

Table 3 Analysis of correlations between Insulin receptor substrate 1 and Ki-67 expression in primary colorectal cancer

Groups of patients		IRS-1 – Ki-67		
		<i>n</i>	<i>P</i> value	<i>r</i>
All		102	0.589	0.054
Age (yr)	≤ 60	32	0.291	-0.193
	> 60	68	0.147	0.178
Sex	Male	55	0.893	0.018
	Female	46	0.517	0.098
Localization	Rectum	45	0.662	-0.067
	Colon	56	0.285	0.145
HP-type	A	85	0.751	0.035
	MA	17	0.239	-0.302
G	G2	71	0.845	0.024
	G3	30	0.714	0.070
T	pT1 + pT2	8	0.043	0.723 ^a
	pT3 + pT4	94	0.973	-0.004
N	pN (-)	49	0.471	0.105
	pN (+)	53	0.999	0.000

Associations were analyzed by Spearman's correlation rank test.

^a*P* < 0.05; *n*: Number of cases; *r*: Correlation coefficient; A: Adenocarcinoma; MA: Mucinous adenocarcinoma; G2: Moderately differentiated; G3: Poorly differentiated; N (-): Negative lymph node invasion; N (+): Positive lymph node invasion.

exerts synergistic effect with antiapoptotic Bcl-xL protein to enable the escape of tumor cells from death signals and continue abnormal proliferation. The expression of Bax protein in more advanced tumor can be independently regulated for example by the oxidative stress^[34].

Role of insulin and IGF-1 signaling in promoting cell growth and proliferation is well established but in present study we did not find evidences for the association between expression of IRS-1 and increased cell proliferation (assessed by Ki-67 positivity), except cases of smaller tumors (pT1 + pT2). Interestingly, in the same groups of patients, IRS-1 expression was positively correlated with antiapoptotic Bcl-xL. Thus, it can be concluded that IRS-1 could promote proliferation and survival signals or activate apoptotic signals in tumor cells, depending on the microenvironmental conditions such as availability of oxygen or nutrients. On the other hand, in our previous study on breast cancer, we found that IRS-1 was positively correlated with Ki-67 in ERα-positive primary tumors and negatively correlated in ERα-negative tumors^[35]. These data could suggest that IRS-1 can promote enhanced proliferation primarily in steroid-dependent cells.

We also observed decreased IRS-1 expression in poorly differentiated, high-grade colorectal tumors and in adenocarcinoma with mucosal component cases. This observation is consistent with other study showing that IRS-1 is expressed at low levels or absent in undifferentiated and mucinous CRCs^[2]. A similar downregulation of IRS-1 expression was observed in non-small cell lung cancer where loss of IRS-1 occurred more frequently in stage IB than in IA tumors and was more frequently observed in squamous cell carcinoma^[36]. The loss of IRS-1 expression at some stage during malignant transformation could suggest that IRS-1-dependent signals play a significant role in the early, but not advanced, stages of tumor development. In line with previous studies, we found that IRS-1 expression is more prevalent in more differentiated tumors, but others have shown that despite this fact IRS-1 expression was also correlated with markers of biological aggressiveness, including Ki-67, p53, and cytoplasmic beta-catenin^[2].

The overall goal of this work was to examine the expression of IRS-1 in CRC and analyze its associations with proliferation and apoptotic markers Ki-67, Bax and Bcl-xL in relation to clinicopathologic features. Our data suggest that (1) IRS-1 expression is

Table 4 Analysis of correlations between Bax and Bcl-xL expression in primary colorectal cancer

Groups of patients		Bax-Bcl-xL		
		<i>n</i>	<i>P</i> value	<i>r</i>
All		115	< 0.001	0.556 ^a
Age(yr)	≤ 60	39	< 0.001	0.575 ^a
	> 60	74	< 0.001	0.530 ^a
Sex	Male	60	< 0.001	0.453 ^a
	Female	54	< 0.001	0.643 ^a
Localization	Rectum	48	< 0.001	0.490 ^a
	Colon	66	< 0.001	0.594 ^a
HP-type	A	93	< 0.001	0.546 ^a
	MA	22	0.013	0.519 ^a
G	G2	80	< 0.001	0.523 ^a
	G3	34	< 0.001	0.543 ^a
T	pT1 + pT2	10	0.537	0.222
	pT3 + pT4	105	< 0.001	0.577 ^a
N	pN (-)	55	< 0.001	0.509 ^a
	pN (+)	60	< 0.001	0.582 ^a

Associations were analyzed by Spearman's correlation rank test.

^a*P* < 0.05; *n*: Number of cases; *r*: Correlation coefficient; A: Adenocarcinoma; MA: Mucinous adenocarcinoma; G2: Moderately differentiated; G3: Poorly differentiated; N (-): Negative lymph node invasion; N (+): Positive lymph node invasion.

more prevalent in more differentiated tumors, and (2) IRS-1 expression is correlated with both proapoptotic Bax and antiapoptotic Bcl-xL proteins. The first observation indicates that loss of IRS-1 in CRC may be considered as potential marker for poor differentiation and more aggressive phenotype. The second observation aligns with published evidence suggesting a potential diverse role of -insulin receptor IGF-1R/IRS-1 signaling in regulating apoptotic processes^[23].

While this study is not conclusive, it provides a good starting point for discussion regarding interactions and functional dependence between IRS-1, Bax and Bcl-xL in CRC. In the future, the assessment of IRS-1 expression could be used to evaluate individual patient prognosis and might offer new insights into developing more efficient treatment strategies and identify patients who are most likely to respond to targeted therapies, for example IGF-1R inhibition.

ARTICLE HIGHLIGHTS

Research background

Insulin receptor substrate 1 (IRS-1), a signaling molecule involved in cell proliferation, survival and metabolic responses has been implicated in carcinogenic processes in various cellular and animal models. However, the role of IRS-1 in human colorectal cancer (CRC) biology and its value as a clinical CRC biomarker has not been well defined.

Research motivation

CRC is the third most common cancer diagnosed across the world. Despite effective prevention and screening methods CRC represents one of the most common causes of cancer-related deaths. Most of the research is aimed at finding new prognostic factors or therapeutic strategy in order to reduce high CRC-related mortality.

Research objectives

This study evaluated if and how IRS-1 expression and its associations with the apoptotic and proliferation tumor markers, Bax, Bcl-xL and Ki-67 are related to clinicopathological features in human CRC, *i.e.*, patient age, sex, primary localization of tumor, histopathological type, grading, staging and lymph node spread.

Research methods

We retrospectively collected data from 127 patients with primary CRC who underwent radical surgery with lymph node dissection. We analyzed the expressions of IRS-1, Bax, Bcl-xL and Ki-67 proteins using immunohistochemical methods. Correlations between variables were examined by Spearman rank correlation test and Fisher exact test with a level of significance at $P < 0.05$.

Research results

Immunohistochemical analysis revealed weak cytoplasmatic staining for IRS-1 in 66 CRC sections and strong cytoplasmatic staining in 61 cases. IRS-1 expression at any level in primary CRC was associated with tumor grade (69% in moderately differentiated tumors, G2 *vs* 31% in poorly differentiated tumors, G3) and with histological type (81.9% in adenocarcinoma *vs* 18.1% in adenocarcinoma with mucosal component cases). Strong IRS-1 positivity was observed more frequently in adenocarcinoma cases (95.1%) and in moderately differentiated tumors (85.2%). We also found different relationships between IRS-1 expression and both Bax and Bcl-xL proteins depended on clinicopathological parameters. Further analysis of the data revealed no significant correlation between expression of IRS-1 and proliferation marker Ki-67, excluding early stage tumors, where the correlation was positive and on a high level ($P = 0.043$, $r = 0.723$).

Research conclusions

Our study adds to a growing corpus of research showing that (1) IRS-1 expression is more prevalent in more differentiated tumors, and our data indicate that (2) IRS-1 expression is correlated with both proapoptotic Bax and antiapoptotic Bcl-xL proteins.

Research perspectives

Further research on this topic might extend the knowledge on the interactions and functional dependence between IRS-1 and apoptotic markers in CRC. In the future, the assessment of IRS-1 expression could be used to evaluate individual patient prognosis and might offer new insights into developing more efficient treatment strategies and identify patients who are most likely to respond to targeted therapies, for example the insulin-like growth factor 1 receptor inhibition.

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Retrospective Study

Enhancement parameters of contrast-enhanced computed tomography for pancreatic ductal adenocarcinoma: Correlation with pathologic grading

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Abstract

BACKGROUND

Pancreatic ductal adenocarcinoma (PDA) is a malignancy with a high mortality rate and short survival time. The conventional computed tomography (CT) has been worldwide used as a modality for diagnosis of PDA, as CT enhancement pattern has been thought to be related to tumor angiogenesis and pathologic grade of PDA.

AIM

To evaluate the relationship between the pathologic grade of pancreatic ductal adenocarcinoma and the enhancement parameters of contrast-enhanced CT.

METHODS

In this retrospective study, 42 patients (Age, mean \pm SD: 62.43 \pm 11.42 years) with PDA who underwent surgery after preoperative CT were selected. Two radiologists evaluated the CT images and calculated the value of attenuation at the aorta in the arterial phase and the pancreatic phase ($VA_{arterial}$ and $VA_{pancreatic}$) and of the tumor ($VT_{arterial}$ and $VT_{pancreatic}$) by finding out four regions of interest. Ratio between the tumor and the aorta enhancement on the arterial phase and the pancreatic phase ($TAR_{arterial}$ and $TAR_{pancreatic}$) was figured out through dividing $VT_{arterial}$ by $VA_{arterial}$ and $VT_{pancreatic}$ by $VA_{pancreatic}$. Tumor-to-aortic enhancement fraction (TAF) was expressed as the ratio of the difference between attenuation of the tumor on arterial and parenchymal images to that between attenuation of the aorta on arterial and pancreatic images. The Kruskal-Wallis analysis of variance and Mann-Whitney *U* test for statistical analysis were used.

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RESULTS

Forty-two PDAs (23 men and 19 women) were divided into three groups: Well-differentiated ($n = 13$), moderately differentiated ($n = 21$), and poorly differentiated ($n = 8$). TAF differed significantly between the three groups ($P = 0.034$) but $TAR_{arterial}$ ($P = 0.164$) and $TAR_{pancreatic}$ ($P = 0.339$) did not. The median value of TAF for poorly differentiated PDAs (0.1011; 95%CI: 0.01100-0.1796) was significantly higher than that for well-differentiated PDAs (0.1941; 95%CI: 0.1463-0.3194).

CONCLUSION

Calculation of TAF might be useful in predicting the pathologic grade of PDA.

Key words: Computed tomography; Pancreatic ductal carcinoma; Diagnostic imaging; Clinical pathology; Neoplasm grading; Prognosis

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Core tip: The conventional computed tomography (CT) has been worldwide used as a modality for diagnosis of pancreatic ductal adenocarcinoma (PDA). In this study, the tumor-to-aortic enhancement fraction (TAF) values were statistically different among the well differentiated group, the moderately differentiated group and the poorly differentiated group ($P < 0.05$). It has been reported that $TAR_{arterial}$ and $TAR_{pancreatic}$ are related to histological finding of PDA, but in our study, there were no significant differences in $TAR_{arterial}$ and $TAR_{pancreatic}$ among the three groups. TAF can be obtained with conventional pancreatic CT, without additional radiation exposure and processing time, and this simple method could be useful for predicting prognosis of PDA.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is a malignancy with a high mortality rate and short survival time^[1]. Several important prognostic factors including tumor size, lymph node status, pathological grading and differentiation of the tumor influence survival in patients with PDA^[2]. The pathological grade of adenocarcinoma is associated with the intratumor microvessel density (MVD)^[3]. The process of neoangiogenesis is mediated by tumor angiogenic factors. Adenocarcinomas that develop in various organs tend to have a characteristic neovascularization pattern^[4-6].

Computed tomography (CT) is an imaging modality used for evaluating tumors. The degree of CT enhancement is thought to be dependent upon the increase or decrease of intratumor MVD^[7]. Some reports have described the relationship between CT enhancement, tumor angiogenesis, and the pathological grade of PDA^[8]. It was reported that the degree of CT enhancement was directly proportional to the pathological grade of lung cancer but inversely proportional to that of PDA^[8,9]. However, to date, few quantitative studies have compared CT enhancement parameters and the pathologic grade of PDA^[10,11]. Therefore, the aim of this study was to investigate the relationship between various CT enhancement parameters and the pathologic grade of PDA.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the institutional review board and the requirement for informed consent was waived. We conducted a computerized search

of electronic medical records for patients with PDA. Forty-eight patients underwent surgery following CT examination from October 2012 to June 2017. We excluded 6 patients because they did not undergo arterial and pancreatic phase CT. A total of 42 patients were enrolled in our study. Forty-two patients with PDA (head and uncinate process: 30, body: 11, tail: 1) were treated using Whipple's procedure ($n = 6$), pylorus preserving pancreaticoduodenectomy ($n = 28$), and distal pancreatectomy ($n = 8$).

CT examination

All CT images were obtained with two 128-channel multi-detector scanners (Siemens SOMATOM Definition AS and Flash, Siemens Healthcare, Erlangen, Germany). The CT parameters were as follows: Slice thickness, 3–5 mm; field of view (FOV), 50 cm × 50 cm; matrix, 512 × 512; beam collimation, 128 mm × 0.625 mm; beam pitch, 0.7; gantry rotation time, 0.5 s; tube voltage, 100–120 kV; and automated dose modulation with a maximum allowable tube current set at 200 mA.

Each patient received 120–150 mL of iohexol 300 (300 mg iodine) (Bonorex 300; Central Medical Service, Seoul, South Korea). An automatic power injector operating at an injection rate of 3.5 mL/s was used. The arterial, pancreatic, and late phase images were obtained with delays of 40 s, 65 s, and 105 s, respectively, after the injection of the contrast agent.

Imaging analysis

Two radiologists, blinded to the clinical data, performed consensual analysis of the axial CT images on a picture archiving and communication system (PACS; G3, Infinitt Healthcare, Seoul, South Korea). The CT attenuation values [Hounsfield Unit (HU)] of the tumor were measured by drawing circular regions of interest (ROIs) on the arterial and pancreatic phases. The attenuation values of the tumor were analyzed in the arterial (VT_{arterial}) and pancreatic phases ($VT_{\text{pancreatic}}$) and expressed in HU; visible necrosis, adjacent pancreatic parenchyma, and large vessels were excluded^[12]. The same ROIs were reproduced at the aorta of the corresponding images, which measured the ROI of the tumor, the attenuation value of the aorta in the arterial phase (VA_{arterial}), and the attenuation value of the aorta in the pancreatic phase ($VA_{\text{pancreatic}}$).

The enhancement parameters, *i.e.*, the tumor-to-aorta enhancement ratios of the arterial (TAR_{arterial}) and pancreatic phases ($TAR_{\text{pancreatic}}$) were the division of VT_{arterial} to VA_{arterial} and $VT_{\text{pancreatic}}$ to $VA_{\text{pancreatic}}$, respectively. $TAR_{\text{arterial}} = VT_{\text{arterial}} / VA_{\text{arterial}}$; $TAR_{\text{pancreatic}} = VT_{\text{pancreatic}} / VA_{\text{pancreatic}}$.

The tumor-to-aortic enhancement fraction (TAF) represents the ratio of difference between the attenuation of the tumor on arterial and parenchymal images to the difference between the attenuation of the aorta on arterial image and pancreatic images. The difference in tumor enhancement between the arterial and pancreatic phases (DT) was calculated by subtracting VT_{arterial} from $VT_{\text{pancreatic}}$ ^[13]. The difference in aortic washout between the arterial and pancreatic phases (DA) was calculated by subtracting $VA_{\text{pancreatic}}$ from VA_{arterial} (Figure 1A and 1B). Thereafter, TAF was calculated by dividing DT by DA. Three equations can be summarized as follows: $DT = VT_{\text{pancreatic}} - VT_{\text{arterial}}$; $DA = VA_{\text{arterial}} - VA_{\text{pancreatic}}$; $TAF = DT/DA = (VT_{\text{pancreatic}} - VT_{\text{arterial}}) / (VA_{\text{arterial}} - VA_{\text{pancreatic}})$.

Statistical analysis

Statistical analyses were performed with SPSS software (SPSS Statistics for Windows, version 20.0; IBM Corp, Armonk, NY, United States). The Kruskal-Wallis analysis of variance (ANOVA) and the Mann-Whitney *U* test were used to evaluate differences among the three groups, *i.e.*, poorly, moderately, and well-differentiated pancreatic tumors^[14]. The patients' age, sex, tumor size, lesion location, TAR_{arterial} , $TAR_{\text{pancreatic}}$, and TAF were compared. Moreover, receiver operating characteristic (ROC) analysis was used to compare the diagnostic performance of TAR_{arterial} , $TAR_{\text{pancreatic}}$, and TAF for predicting the pathologic grade of PDA. $P < 0.05$ was considered statistically significant.

RESULTS

The study included 23 men and 19 women with a mean age of 62.43 years (SD: 11.42; range: 34–85 years). The 42 lesions investigated in our study were located in the pancreatic head and uncinate process ($n = 30$), body and neck ($n = 11$), and tail ($n = 1$).

A total of 42 PDAs were categorized into three groups: (1) The well-differentiated group (WD) ($n = 13$); (2) The moderately differentiated group (MD) ($n = 21$); and (3)

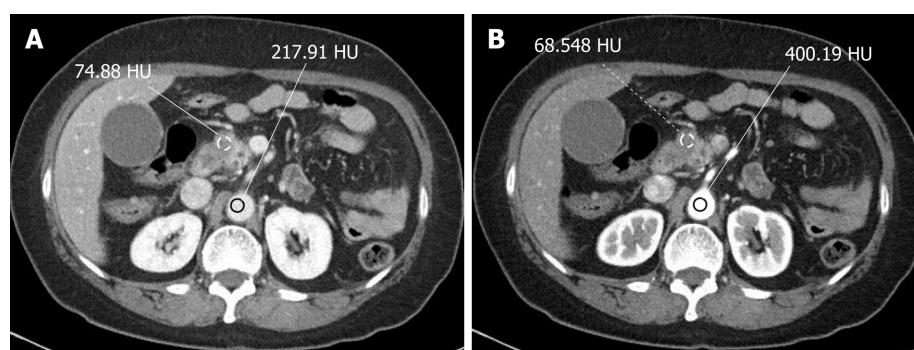


Figure 1 Axial computed tomography images on arterial phase (A) and pancreatic phase (B) of a 68-year-old woman, who was classified in the poorly differentiated group. A: Computed tomography (CT) attenuation values [Hounsfield Unit (HU)] of the tumor in the arterial phase ($VT_{arterial}$) and the aorta in the arterial phase ($VA_{arterial}$) were measured by drawing two separate circular regions of interest in the aorta and pancreas on arterial phase image; B: CT HU of the tumor in a pancreatic phase ($VT_{pancreatic}$) and the aorta in the pancreatic phase ($VA_{pancreatic}$) were measured in the aorta and pancreas on the pancreatic phase 65 s after the injection of the contrast agent.

The poorly differentiated group (PD) ($n = 8$). The size of the lesions ranged from 1.5 to 7 cm for WD lesions, 1.8 to 5.0 cm for MD lesions, and 2.2 to 13.0 cm for PD lesions, respectively. There were no significant differences in size and location of the lesion among the WD, MD, and PD groups ($P \leq 0.076$). Patient characteristics are summarized in Table 1.

There were no significant differences in values of $VT_{arterial}$, $VT_{pancreatic}$, $VA_{arterial}$, $VA_{pancreatic}$, DT, and DA among the three groups (Table 2). Moreover, there was no significant difference in the value of $TAR_{arterial}$ among the WD (mean: 0.26, 95%CI: 0.1903-0.3340), MD (mean: 0.27; 95%CI: 0.2284-0.3122), and PD groups (mean: 0.19; 95%CI: 0.1295-0.2465). There was no significant difference in the value of $TAR_{pancreatic}$ among the WD (mean: 0.45, 95%CI: 0.3493 to 0.5435), MD (mean: 0.48; 95%CI: 0.3988-0.5557), and PD groups (mean: 0.37; 95%CI: 0.2724-0.4759) ($P < 0.0001$) (Table 3).

The value of TAF was statistically different among the three groups; WD (median and mean, 0.19 and 0.28, respectively; 95%CI: 0.1370-0.4289), MD (median and mean, 0.17 and 0.19, respectively; 95%CI: 0.1377-0.2468), and PD (median and mean, 0.10 and 0.10, respectively; 95%CI: 0.02948-0.1675) ($P < 0.05$).

The diagnostic performances of $TAR_{arterial}$, $TAR_{pancreatic}$, and TAF for the prediction of the pathological grade of PDA are shown in Table 2. The diagnostic performance of TAF ($Az = 0.692$ -0.757) was higher than that of $TAR_{arterial}$ ($Az = 0.509$ -0.71) and $TAR_{pancreatic}$ ($Az = 0.512$ -0.654) for predicting the pathological grade of PDA, although the difference was not statistically significant ($P > 0.093$).

DISCUSSION

The pathological tumor grade is an important prognostic factor of survival in patients with PDA^[2]. PDA has unique characteristics and different CT enhancement patterns (such as lung and renal cancers)^[8,9,12,15,16], based on the proportion of MVD, degree of fibrosis, and residual normal pancreatic tissue.

There were no significant differences in $VT_{arterial}$ and $VT_{pancreatic}$ among the three groups in our study. Several researchers^[8,10] have studied the correlation between CT enhancement parameters and the histological findings of pancreatic adenocarcinomas. Wang *et al*^[8] reported that the pathological grade showed a good correlation with $VT_{pancreatic}$ and MVD. In contrast, Hattori *et al*^[10]'s study on pancreatic ductal cancer reported that $VT_{arterial}$ and $VT_{pancreatic}$ were negatively correlated with the degree of fibrosis. $VT_{arterial}$ showed a significant correlation with vascular endothelial growth factor and MVD but $VT_{pancreatic}$ was not correlated with MVD. Hattori *et al*^[10] reported that $TAR_{arterial}$ was positively correlated with MVD and negatively correlated with the extent of fibrosis. However, our findings demonstrate that there were no significant differences in $TAR_{arterial}$ and $TAR_{pancreatic}$ among the three groups.

There were no significant differences in the values of DT and DA among the WD, MD, and PD groups. Aortic enhancement curves showed a decreased slope from the arterial to the pancreatic phases, after the arterial phase and tumor enhancement curves showed an increased slope. However, the degree of aortic enhancement is influenced by the dose of the contrast media, rate of injection, appropriate timing of

Table 1 Distribution of patient characteristics, lesion size and location

	WD group (n = 13)	MD group (n = 21)	PD group (n = 8)	P value
Age, mean ± SD	61.69 ± 10.13	64.85 ± 11.95	55.25 ± 10.17	0.051
Sex				0.542
Male	8	12	3	
Female	5	9	5	
Size, mean ± SD	2.75 ± 1.61	2.95 ± 0.91	4.23 ± 3.62	0.114
Location				0.076
Head and uncinate process	10	17	3	
Body and neck	3	4	4	
Tail	0	0	1	

Data are presented as mean ± SD. WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated.

Table 2 Diagnostic performance of computed tomography parameters for the prediction of pathological grading

	WD-MD (n = 34) vs PD (n = 8)				WD (n = 13) vs MD-PD (n = 8)			
	Az	SD	P value	95%CI	Az	SD	P value	95%CI
TAR _{arterial}	0.71	0.101	0.467	0.549 - 0.839	0.509	0.107	0.989	0.351 - 0.667
TAR _{pancreatic}	0.654	0.112	0.742	0.492 - 0.794	0.512	0.0993	0.196	0.353 - 0.669
TAF	0.757	0.102	0.428	0.601 - 0.876	0.692	0.0829	0.093	0.531 - 0.825

Data are presented as mean ± SD (median). CT: Computed tomography; WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated group; TAR_{arterial} and TAR_{pancreatic}: Tumor-to-aorta enhancement ratio in the arterial and pancreatic phases; TAF: Tumor-to-aortic enhancement fraction.

contrast-enhanced imaging, heart rate and cardiac output of the patient, weight, and age^[17]. The renal cancer study divided tumor enhancement with aortic enhancement to correct these intrinsic factors^[15].

Finally, the TAF values were statistically different among the WD (median and mean, 0.19 and 0.28, respectively; 95%CI: 0.1370-0.4289), MD (median and mean, 0.17 and 0.19, respectively; 95%CI: 0.1377-0.2468), and PD (median and mean, 0.10 and 0.10, respectively; 95%CI: 0.02948-0.1675) ($P < 0.05$). Perfusion imaging demonstrates blood flow in the target organ using single-photon emission computed tomography, CT, and magnetic resonance imaging. Perfusion CT can identify vascularity and fibrosis in the diseased pancreas^[18,19]. Various perfusion CT parameters can be generated by postprocessing the CT data. Perfusion CT has a smaller FOV, requires additional radiation exposure, and processing time. However, TAF may be obtained with conventional pancreatic CT, without additional radiation exposure and processing time, and is more useful for practical staging than perfusion CT parameters.

There are several limitations to our study. First, we drew two similar ROIs at the aorta and tumor for minimizing intraobserver variation: Two radiologists consensually reviewed PDA lesions in the arterial and pancreatic phases. Therefore, we could not ascertain the inter or intraobserver variations. Second, this study had an inherent bias owing to its retrospective design. Third, our sample size was small, which made it difficult to obtain statistically significant data. Finally, there was no statistically significant difference, but the number of patients with MD PDAs was greater than patients with PD and WD PDAs. Therefore, prospective studies with large populations are needed in the future, to overcome these limitations.

Table 3 Differences in computed tomography parameters among the well-differentiated, moderately differentiated, and poorly differentiated group groups

	WD (<i>n</i> = 13)	MD (<i>n</i> = 21)	PD (<i>n</i> = 8)	<i>P</i> value
VT _{arterial}	69.01 ± 25.16 (64.22)	75.23 ± 19.69 (75.41)	59.80 ± 29.47 (56.30)	0.145
VT _{pancreatic}	85.34 ± 23.20 (76.08)	90.58 ± 21.99 (90.58)	73.19 ± 39.88 (69.01)	0.184
VA _{arterial}	280.71 ± 66.66 (262.74)	290.37 ± 61.12 (279.38)	320.04 ± 86.26 (311.20)	0.496
VA _{pancreatic}	201.73 ± 55.81 (200.00)	200.85 ± 43.82 (209.44)	188.28 ± 46.94 (195.43)	0.683
DT	16.32 ± 6.48 (13.65)	15.34 ± 8.06 (13.97)	13.39 ± 13.25 (10.16)	0.678
DA	78.98 ± 49.68 (61.54)	89.51 ± 34.84 (91.01)	131.76 ± 58.68 (120.79)	0.077
TAR _{arterial}	0.26 ± 0.12 (0.23)	0.27 ± 0.09 (0.25)	0.19 ± 0.07 (0.20)	0.164
TAR _{pancreatic}	0.45 ± 0.16 (0.36)	0.48 ± 0.17 (0.47)	0.37 ± 0.12 (0.38)	0.339
TAF	0.28 ± 0.24 (0.19)	0.19 ± 0.12 (0.17)	0.10 ± 0.08 (0.10)	0.034

Data are presented as mean ± SD (median). WD: Well-differentiated; MD: Moderately differentiated group; PD: Poorly differentiated group; VT_{arterial} and VT_{pancreatic}: Attenuation value of the tumor in the arterial and pancreatic phases, respectively; VA_{arterial} and VA_{pancreatic}: Attenuation value of the aorta in the arterial and pancreatic phases, respectively; DT and DA: Degree of tumor enhancement and aortic washout between the arterial and pancreatic phases, respectively; TAR_{arterial} and TAR_{pancreatic}: Tumor-to-aorta enhancement ratio in arterial and pancreatic phases, respectively; TAF: Tumor-to-aortic enhancement fraction.

ARTICLE HIGHLIGHTS

Research background

Pancreatic ductal adenocarcinoma (PDA) is a malignancy with a high mortality rate and short survival time. The conventional computed tomography (CT) has been worldwide used as a modality for diagnosis of PDA. Also, it has been widely accepted that CT enhancement pattern is related to tumor angiogenesis and pathologic grade of PDA.

Research motivation

Although there is other modality, like perfusion CT that provide information about vascularity and fibrosis in the diseased pancreas, it has a smaller FOV, requires additional radiation exposure, and processing time. So, if there is any CT parameter that can predict pathologic grade of PDA, it would be useful for predicting prognosis of PDA using conventional CT.

Research objectives

In this study, we aimed to evaluate the relationship between the pathologic grade of pancreatic ductal adenocarcinoma and the enhancement parameters of contrast-enhanced CT.

Research methods

In this retrospective study, 42 patients with PDA who underwent surgery after preoperative CT were selected. Two radiologists evaluated the CT images and calculated the value of attenuation at the aorta in the arterial phase and the pancreatic phase (VA_{arterial} and VA_{pancreatic}) and of the tumor (VT_{arterial} and VT_{pancreatic}) by finding out four regions of interest. Ratio between the tumor and the aorta enhancement on the arterial phase and the pancreatic phase (TAR_{arterial} and TAR_{pancreatic}) was figured out through dividing VT_{arterial} by VA_{arterial} and VT_{pancreatic} by VA_{pancreatic}. Tumor-to-aortic enhancement fraction (TAF) was expressed as the ratio of the difference between attenuation of the tumor on arterial and parenchymal images to that between attenuation of the aorta on arterial and pancreatic images.

Research results

A total of 42 PDAs were categorized into three groups: Well-differentiated (*n* = 13), moderately differentiated (*n* = 21), and poorly differentiated (*n* = 8). TAF differed significantly between the three groups (*P* = 0.034) but TAR_{arterial} (*P* = 0.164) and TAR_{pancreatic} (*P* = 0.339) did not. The value of TAF was statistically different among the

three groups ($P < 0.05$).

Research conclusions

TAF was statistically different among the three pathologic grade groups. So, the TAF might be correlated with histological finding of PDA. Therefore, calculation of TAF using conventional CT might be useful in predicting the pathologic grade of PDA.

Research perspectives

The conventional CT has been useful modality for diagnosis of PDA. In our study, we suggest the CT enhancement parameter, TAF, could be used as a value for predicting pathologic grade of PDA. The pathologic grade is related to prognosis of PDA, then we can use conventional CT not only for diagnosis, but also for predicting pathologic grade and prognosis of PDA. Also, TAF may be obtained with conventional pancreatic CT, without additional radiation exposure and processing time, and is more useful for practical staging than perfusion CT parameters.

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Observational Study

Detection of reflux-symptom association in children with esophageal atresia by video-pH-impedance study

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Institutional review board statement: The study was

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Abstract

BACKGROUND

Children with esophageal atresia (EA) have risk of gastroesophageal reflux

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disease (GERD), suggesting reflux monitoring for prompt management.

AIM

To evaluate GERD in children with EA and specific symptom association from combined Video with Multichannel Intraluminal Impedance and pH (MII-pH) study.

METHODS

Children diagnosed with EA with suspected GERD and followed up at King Chulalongkorn Memorial Hospital between January 2000 and December 2018 were prospectively studied. All underwent esophagogastroduodenoscopy with esophageal biopsy and Video MII-pH study on the same day. Symptoms of GERD which included both esophageal and extra-esophageal symptom were recorded from video monitoring and abnormal reflux from MII-pH study based on the statement from the European Paediatric Impedance Group. Prevalence of GERD was also reported by using histopathology as a gold standard. Endoscopic appearance was recorded using Los Angeles Classification and esophagitis severity was graded using Esophisto criteria.

RESULTS

Fifteen children were recruited with age of 3.1 (2.2, 9.8) years (40%, male) and the common type was C (93.3%). The symptoms recorded were cough (75.2%), vomiting (15.2%), irritability or unexplained crying (7.6%) and dysphagia (1.9%) with the symptom-reflux association of 45.7%, 89%, 71% and 0%, respectively. There were abnormal endoscopic appearance in 52.9%, esophagitis in 64.7% and high reflux score in 47.1%. Video MII-pH study has high diagnostic value with the sensitivity, specificity and accuracy of 72.7%, 100% and 82.4%, respectively.

CONCLUSION

Prevalence of GERD in children with EA was high. Video MII-pH study to detect GERD in children with EA had high diagnostic value with the trend of specific symptom association.

Key words: Gastroesophageal reflux disease; Esophageal atresia; Children; Impedance pH study; Video; Symptom association

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Core tip: This was a cross sectional study with 15 patients diagnosed with esophageal atresia (EA) and suspected gastroesophageal reflux disease (GERD). Combined Video Multichannel Intraluminal Impedance and pH study has a good diagnostic accuracy to diagnose GERD in these children and there was a trend of specific symptom-reflux association in children diagnosed with EA.

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INTRODUCTION

Esophageal atresia (EA) is a common digestive malformation occurring in 1:2400-4500 births. Improvements in operative and medical care enable them to have longer life expectancy but suffering from comorbidities including pathological gastroesophageal reflux (GER). Although GER disease (GERD) is defined as the reflux of gastric contents lead to troublesome symptoms^[1], unlike other children, EA children with GERD could be asymptomatic or present with extraesophageal symptoms^[2]. Therefore, the recent European Society of Paediatric Gastroenterology, Hepatology and Nutrition and

North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN and NASPGHAN) guideline for EA children recommended to routinely prescribe proton pump inhibitors (PPIs) up to the first year of life and monitor reflux episodes using combined esophageal Multichannel Intraluminal Impedance and pH (MII-pH) monitoring and/or endoscopy at time of discontinuation and during long-term follow up^[2].

The prevalence of GERD in EA patients varied from 22%-45%, depending on the diagnostic criteria^[3-7]. Esophageal histopathology is the gold standard test to diagnose pathological reflux but it has low sensitivity compared to others^[8]. MII-pH monitoring is one of the best diagnostic tools for GERD as its ability to detect the frequency, height and type of reflux event. However, the normal value of reflux event in children is scarce hence the data should be interpreted with caution. The international guideline^[9] recommended using MII-pH study to correlate persistent troublesome symptoms with reflux episodes instead. Using video monitoring, symptoms should be recorded more precisely with time of reflux than by caregivers in EA children. We aim to study the prevalence of GERD and the symptom correlation in EA children using Video MII-pH.

MATERIALS AND METHODS

Patients

Children diagnosed with EA who received esophageal anastomosis and regularly followed up at King Chulalongkorn Memorial Hospital between 1 January 2000 and 31 December 2018 were recruited. This cross sectional study has been approved by the Institutional Review Board of Chulalongkorn University (IRB number 243/61). Written informed consent was obtained from all parents and informed assent from patients over 7 years old before any procedure was performed.

Data collection and outcome measurements

All patients were admitted. Detailed demographic data, comorbidities, signs and symptoms followed the international guidelines^[9] (Table 1), and previous investigations were collected by investigator's interview and medical records.

Esophagogastroduodenoscopy

On the following day, all patients were nil by mouth for at least 6 h before esophagogastroduodenoscopy (EGD) under general anesthesia. Esophageal biopsy was performed at 3-5 cm above z-line for at least 2 pieces.

Combined Video MII-pH monitoring

The age appropriate catheter (Pediatric ZandorpH catheter with 1 Antimony and 6 impedance rings with 2 cm interval, Laborie, The Netherlands) was inserted after EGD and under general anesthesia. When the patient woke up, the catheter position was adjusted to place pH sensor at 2 vertebrae above the diaphragm from a plain chest x-ray in upright and full inspired position, followed the statement from British Society of Paediatric Gastroenterology, Hepatology and Nutrition (BSPGHAN) motility working group^[10]. All chest X-ray was reviewed by a pediatric gastroenterologist (Sintusek P) to confirm the proper position. Then combined MII-pH machine (Ohmega, Laborie, The Netherlands) was started and monitored for at least 24 h. Video monitoring was done and the MII-pH study was synchronized with the video. All signs and symptoms of GERD according to ESPGHAN and NASPGHAN guidelines^[9] were recorded by instructed caregivers while the main investigator (Maholarnkij S) independently recorded them from the video record. Carbonated drink, juices and acidic foods were prohibited from the patient during the monitoring.

Combined Video MII-pH analysis

Tracing from MII-pH study results were initially analyzed using Laborie automated analysis software (MMS database software, The Netherlands) and revised visually and manually analyzed by the pediatric gastroenterologist (Sintusek P). The criteria for all parameters followed the position statement by BSPGHAN Motility Working Group^[10] and the normal values of reflux followed the European Paediatric Impedance Group (EURO-PIG)^[11]: (1) Reflux is classified as acid (pH < 4), weakly acid (pH 4-7), and weakly alkaline (pH > 7); (2) Reflux index (RI) is defined as percentage of time with esophageal pH through the pH sensor above lower esophageal sphincter < 4. We considered pathological acid reflux if RI > 3% in children aged > 1 year; (3) Number of

Table 1 Symptoms and signs that may be associated with gastroesophageal reflux in infants and children^[9]

Symptoms	Signs
General	General
Discomfort/irritability ¹	Dental erosion
Failure to thrive	Anemia
Feeding refusal	
Dystonic neck posturing (Sandifer syndrome)	
Gastrointestinal	Gastrointestinal
Recurrent regurgitation with/without	Esophagitis
vomiting in the older children	Esophageal stricture
Heartburn/chest pain ²	Barrett esophagus
Epigastric pain ²	
Hematemesis	
Dysphagia/odynophagia	
Airway	Airway
Wheezing	Apnea spells
Stridor	Asthma
Cough	Recurrent pneumonia associated with aspiration
Hoarseness	Recurrent otitis media

¹If excessive irritability and pain is the single manifestation, it is unlikely to be related to gastroesophageal reflux disease.

²Typical symptoms of gastroesophageal reflux disease in older children.

refluxes is defined as the number of retrograde bolus movements that ≥ 100 episodes in infant and ≥ 70 episodes in children aged > 1 year considered to be pathologic; (4) Symptom index is defined as the following formula: [Reflux-related symptom events/the total symptoms events] $\times 100$. It was considered positive if the value $\geq 50\%$; (5) Symptom sensitivity index is defined as the following formula: [Number of symptom-associated reflux/total number of reflux episodes] $\times 100$. It is positive if the value $\geq 10\%$ for each symptom; (6) Symptom association probability (SAP) is from complex statistical calculation by the machine in for the symptom correlation in each 2-min window of the study. SAP values are considered positive if the value $\geq 95\%$; (7) Esophageal acid clearance is defined as the time from drop in esophageal pH at pH channel < 4 to restoration of pH above ≥ 4 ; (8) Mean bolus clearing time is defined as the mean time in seconds required for the impedance, distal channel, to go back to the initial value after an episode of reflux; and (9) Longest reflux period (min) is defined as the total time that esophageal pH above pH channel < 4 .

Esophageal gross finding

Endoscopic appearance was recorded by the pediatric gastroenterologist using Los Angeles Classification^[12]. Grade A indicated ≥ 1 mucosal breaks confined to the mucosal folds ≤ 5 mm length. Grade B indicated ≥ 1 mucosal breaks > 5 mm, but not continuous between the 2 mucosal folds. Grade C indicated continuous mucosal breaks $< 75\%$ of the esophageal circumference. Grade D indicated mucosal breaks which involves $\geq 75\%$ of the esophageal circumference.

Esophageal histopathology finding

Histopathological severity were reported by a pathologist using modified Esohisto criteria^[13]. The criteria included the first 4 in these 6 parameters: (1) Basal cell layer hyperplasia: Measure thickness of basal cell layer in micrometers and express as a proportion (%) of total epithelial thickness ($\times 10$). The severity score defined as 0 ($< 15\%$), 1 ($15\%-30\%$), and 2 ($> 30\%$); (2) Papillary elongation: measure papillary length in micrometers and express as a proportion (%) of total epithelial thickness ($\times 10$). The severity score defined as 0 ($< 50\%$), 1 ($50\%-75\%$), and 2 ($> 75\%$); (3) Dilatation of

intercellular spaces: identified as irregular round dilatations or diffuse widening of intercellular space ($\times 40$). The severity score defined as 0 (absent), 1 (small; diameter < 1 lymphocyte), and 2 (large; diameter ≥ 1 lymphocyte); (4) Intraepithelial eosinophils: Counted in the most affected high-power field ($\times 40$). The severity score defined as 0 (absent), 1 (1-2 cells), and 2 (> 2 cells); (5) Intraepithelial neutrophils: Counted in the most affected high-power field ($\times 40$). The severity score defined as 0 (absent), 1 (1-2 cells), and 2 (> 2 cells); and (6) Intraepithelial mononuclear cells: counted in the most affected high-power field ($\times 40$). The severity score defined as 0 (0-9 cells), 1 (10-30 cells), and 2 (> 30 cells).

The sum of severity scores divided by the number of lesion types assessed can be stratified into degree of esophagitis. Score 0-0.25 were indicated normal, score 0.5-0.75 were regarded as mild esophagitis, and score 1 or higher qualified for severe esophagitis.

Statistical analysis

Data were analysed with IBM SPSS statistics 22.0. Demographic data were reported as median (IQR) for numerical variables while percentage or proportion for categorical variables. Diagnostic test was calculated and presented as sensitivity, specificity, accuracy, positive predictive value, and negative predictive value by using esophageal histopathology as the gold standard. Statistical significance between paired continuous variables were calculated by Wilcoxon signed ranks test. χ^2 or Fisher's exact test for categorical variables. Clinically significance were defined as P -value < 0.05 . The statistical review of the study was performed by a biomedical statistician at Department of Statistics Science, Kasetsart University, Thailand, Bangkok, Thailand.

The primary outcome is to study the prevalence of GERD in children with EA using combined Video MII-pH study. The secondary outcome is to evaluate the specific symptom association of GERD in children with EA.

RESULTS

Patient characteristics

There were 15 patients diagnosed with EA recruited into the present study. The median age was 3.08 (range from 1.4 to 12.9) years (40%, male) and the most common type was C (93.3%). Ten (33.3%) patients had at least one comorbidity [cardiac malformations ($n = 9$), renal malformations ($n = 3$), anorectal malformations ($n = 2$), musculoskeletal malformations ($n = 2$), lung hypoplasia ($n = 1$), vertebral anomalies ($n = 1$), others (skin tags, growth hormone deficiency) ($n = 3$)]. Eleven (73.3%) patients underwent esophageal anastomosis since neonate and 4 (26.7%) underwent delayed esophageal anastomosis within the first year of life. Ten patients underwent EGD with esophageal biopsy before and seven of them had reflux esophagitis. Five patients were using PPIs (33%) (omeprazole 1-2 mg/kg per day; $n = 2$, lansoprazole 2-3 mg/kg per day; $n = 3$) and two of these were using prokinetic drugs (13.3%) (domperidone 0.3-0.5 mg/kg/dose every 6-8 h) at the time of recruitment. The medication was continued before MII-pH monitoring due to esophagitis finding from previous endoscopy and histopathology. Fundoplication was performed in two patients because of the pharmacological failure. The most common previously symptom reported were recurrent regurgitation with or without vomiting (60%) and cough (60%). Dental screening was performed in six patients and founded dental erosion in three patients (50%). The most common previously signs documented were esophagitis (53%) and recurrent respiratory tract infection (53%) (Table 2). In these 15 children diagnosed with EA, we got 17 records from Video MII-pH study due to two patients were performed for two times for reassessment during pharmacological therapy.

GERD diagnosed by gross and histopathology

Gross appearance on endoscopic view showed abnormality in 52.9% while esophageal histopathology demonstrated esophagitis in 64.7% of them. Three (37.5%) patients with normal gross appearance had histopathology of reflux esophagitis (Table 3).

GERD diagnosed by combined Video MII-pH study and the symptom association

The median of monitoring period excluding fed periods (hours) was 21.2 (19.3-22.1). The median RI (%), and esophageal clearance (minutes) were 2.7 (0.5-9.5) and 1.4 (0.6-2.5), respectively. There was no statistical significance of the symptom recorded by caregivers and video monitoring that was recorded by primary investigators [2.5 (1-

Table 2 Patient demographic data and characteristics (n = 15)

Characteristics	Median (IQR) or n (%)
Male sex	6 (40)
Age (yr)	3.1 (2.2-9.8)
Weight for height (%)	100 (89.4-104.6)
Previous symptoms	
General	
Discomfort/ Irritability in infants	3
Failure to thrive or weight loss	8
Feeding refusal	3
Total	10 (66.7)
Gastrointestinal	
Recurrent regurgitation with or without vomiting	9
Heartburn or chest pain	1
Epigastric pain	1
Hematemesis	1
Dysphagia, odynophagia	6
Total	11 (73.3)
Airway	
Wheezing, stridor	4
Cough	9
Hoarseness	2
Total	10 (66.7)
Previous signs	
General	
Dental erosion	4
Anemia	2
Total	4 (26.7)
Gastrointestinal	
Esophagitis	8
Esophageal stricture	7
Barret's esophagus	2
Total	12 (80)
Airway	
Asthma	1
Recurrent respiratory tract infection	8
Recurrent otitis media	2
Total	8 (53.3)

EA: Esophageal atresia; GER: Gastroesophageal reflux; IQR: Interquartile range.

4.5) *vs* 3 (1-5), $P = 0.282$]. Using the cut-off value from EURO-PIC for the RI and/ or total reflux time, 47.1% of them considered acid/weakly acid related GERD diagnosis. Other MII-pH study parameters are shown in Table 4. In subgroup analysis, there was no significantly different result of combined Video MII-pH monitoring between using and non-using acid suppression therapy during the monitoring (Table 5).

Table 3 Esophagogastroduodenoscopy findings and biopsy results of children with esophageal atresia after esophageal anastomosis (n = 17)

Classification	n (%)
Los Angeles Classification	
Normal ¹	8 (47.1)
A ²	2 (11.8)
B ³	4 (23.5)
C ⁴	3 (17.6)
Pathology	
Normal	6 (35.3)
Mild esophagitis	2 (11.8)
Severe esophagitis	9 (52.9)

¹One or more mucosal breaks confined to the mucosal folds, each not more than 5 mm in maximum length.

²One or more mucosal breaks more than 5 mm in maximum length, but not continuous between the tops of two mucosal folds.

³Mucosal breaks that are continuous between the tops of two or more mucosal folds, but which involve less than 75% of the esophageal circumference.

⁴Mucosal breaks which involve at least 75% of the esophageal circumference.

Table 4 Parameters used and the analysis result of combined multichannel intraluminal impedance and pH study in children diagnosed esophageal atresia after esophageal anastomosis (n = 17)

Parameters	Median (IQR)
Monitoring period excluding fed periods (h)	21.2 (19.3-22.1)
RI (%)	2.7 (0.5-9.5)
Longest reflux period (min)	20 (5-29)
Esophageal clearance (min)	1.4 (0.6-2.5)
Total reflux (times)	19 (11-36)
Acid	9 (4-14)
Weakly acid	10 (6-15)
Weakly alkaline	0 (0-0)
mean bolus clearance time (s)	14.9 (10.4-19.2)

RI: Reflux index.

The total symptoms recorded from video of all 17 combined Video MII-pH monitoring were cough (67.3%), vomiting (17.3%), irritability or unexplained crying (13.4%) and dysphagia (1.9%). In aspect of symptom association, vomiting was the symptom that mostly associated with reflux followed by irritability or unexplained crying and cough (Table 6).

Diagnostic value of combined Video MII-pH study compared to esophageal histopathology

Using esophageal histopathology as the gold standard for GERD, combined Video MII-pH has high diagnostic value with the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of 72.7%, 100%, 100%, 67% and 82.4%, respectively.

DISCUSSION

The present study demonstrated the high prevalence of GERD in children with EA

Table 5 Parameters used and results from the analysis of combined Video Multichannel Intraluminal Impedance and pH study in children diagnosed esophageal atresia after esophageal anastomosis between using and non-using acid suppression therapy (*n* = 17)

Parameters	Acid suppression therapy (<i>n</i> = 7)	No acid suppression therapy (<i>n</i> = 10)	<i>P</i> value
Monitoring period (h)	21.2 (18.1-24.5)	21.3 (19.5-22.1)	0.696
RI (%)	7.1 (1.4-10)	1.45 (0.3-4.2)	0.24
Longest reflux period (min)	29 (5-96)	16 (4-24)	0.143
Esophageal clearance (min)	2.0 (1.0-3.6)	1.0 (0.5-1.7)	0.261
Total reflux (times)	19.0 (11-46)	19.0 (11-29)	0.66
Acid	10.0 (4-16)	6.5 (3-11)	0.558
Weakly acid	13.0 (4-32)	9.0 (6-14)	0.733
Weakly alkaline	0.0 (0-0)	0.0 (0-1)	0.123
Mean bolus clearance time (s)	11.9 (9.3-16.5)	17.3 (10.4-21.7)	0.242
Number of symptoms (times)	3.0 (1.0-5.0)	4.0 (1.0-7.0)	0.452
SI (%)	25 (0.0-50)	10.5 (0-66.7)	0.84
SSI (%)	1.4 (0-5.3)	3.7 (0.0-17.6)	0.419
SAP (%)	73.9 (0-90.9)	83.9 (0-99.1)	0.649

Present as median (IQR). RI: Reflux index; SAP: Symptom association probability; SI: Symptom index; SSI: Symptom sensitivity index.

Table 6 Symptoms and symptom correlation from video recording in children with esophageal atresia (*n* = 17)

Symptom	Symptoms	Symptom-reflux correlation	Acid	Weakly acid	Non acid	SI	SSI	SAP
Cough	35	16 (45.7)	9	7	0	58.5 (6.2-100)	2.9 (0.3-7.1)	95 (18.9-99.2)
Vomit	9	8 (89)	5	3	0	75 (50-100)	3.9 (2.3-60.7)	99.6 (87.8-99.9)
Irritability or unexplained crying	7	5 (71)	4	1	0	50 (25-50)	3.8 (2.5-3.8)	92.8 (72.9-92.8)
Dysphagia	1	0 (0)	0	0	0	0	0	0
Total	52	29 (55.7)	18	11	0	58.4 (27-100)	3.9 (1.3-7.1)	92.3 (77.4-99.6)

Present as *n* (%) or median (IQR). SI: Symptom index; SSI: Symptom sensitivity index; SAP: Symptom association probability.

based on the gold standard tools, esophageal histopathology and/or combined MII-pH study. Most of them had the previous symptoms suspected GERD in aspect of general, gastrointestinal and respiratory system (Table 1). In this study, combined Video MII-pH study could depict a trend of symptom-reflux association of vomiting, irritability or unexplained crying and cough.

Previous studies supported the high incidence of GERD in children with EA though the different diagnostic tools^[3,4,9,14,15]. Esophageal histopathology is considered the gold standard to detect the early reflux esophagitis while MII-pH study, previous gold-standard test for GERD^[16], had the main limitation in aspect of normal value for age. The strength of MII-pH study is its high accuracy if there is the specific symptom correlation during monitoring. The present study found most children with EA had histopathology result compatible with reflux esophagitis and MII-pH study had high specificity to this reflux esophagitis. Moreover, there is a trend of symptom-reflux correlation of vomiting, irritability or unexplained crying and cough. However, symptom recorded during Video MII-pH study per person was too small to extrapolate that GERD was the cause of these symptoms. Reevaluation of these clinical symptoms with Video MII-pH study after adequate treatment might strongly confirm that the symptom-reflux association for further study. As the prevalence of GERD and

it's complications tended to increase very early, the ESPGHAN-NASPGHAN guidelines for children with EA recommended using PPIs in all EA patients in the neonatal period and should be longer, depending on persistence of GERD. As a result all EA patients should undergo MII-pH study, at least, at the time of discontinuation of PPIs and during long-term follow-up. Furthermore, significant esophageal morbidity in adult with EA is evidenced. The prevalence of Barrett esophagus is at least 4-fold higher among adult population with EA^[17] and the cumulative incidence of esophageal squamous cell carcinoma at fourth decade was 50 times^[18] when compared with general population. Consequently, regular surveillance and follow-up patients with EA and GERD should be included not only MII-pH study but also EGD and esophageal biopsy to optimize therapy so that Barrett esophagus and esophageal carcinoma, hopefully, could be avoided.

In theory, combined Video MII-pH study should provide the accurate symptoms that could be confidently correlated with the reflux from the tracer. We could confirm the more numbers and specificity of the symptom recorded by video monitoring compared to the record from caregivers even though it did not reach statistical significance that might because of low number of subjects. Moreover, the precise time of symptom recorded could increase the symptom association as the machine will count the 2-min window period before a reflux event. However, this precise recording consumes time (more than 3-h recording by a pediatrician per patient) that makes it impossible in routine clinical practice. In the future, real-time Video MII-pH monitoring machine should be developed for children suspected GERD so that clinician will manually correlate the reflux with the real-time symptoms from video monitoring. For the overall symptoms during the MII-pH study, we could use the symptom record from caregivers and manually analyze the suspected symptom correlation by checking the video in case that caregiver might delay recording more than the 2-min window period.

Pathological mechanism of GERD in EA was postulated in many studies. Disruption of vagal denervation, vascular interruption, or traction on the lower esophagus occurred after esophageal corrective procedure could be the risk factors of GERD in children with EA^[19] However, children with isolated tracheoesophageal fistula could have severely esophageal motility before surgical repair that might imply the congenital esophageal dysmotility rather than acquired from surgical correction^[20] Esophagus per se might be the main risk factor of GERD in children with EA. Although the MII-pH study could not evaluate the esophageal motility, the long duration of esophageal clearance and mean bolus clearance time from the present study might reflect the esophageal dysmotility of children with EA. Apart from esophageal dysmotility, the present study demonstrated that mainly GERD in these children was acid reflux in etiology that 3 and 2 of them has no response with PPIs therapy and fundoplication, respectively. More aggressive management could be considered and esophageal motility should be evaluated before surgical fundoplication as this surgery could impair esophageal clearance and worsening the reflux esophagitis.

The strength of the present study is the evaluation of both esophageal histopathology and combined MII-pH study in the meantime. To the best of our knowledge, this is the first study that integrate video recording into the MII-pH study and a pediatrician was the person recording the symptom that might associate with reflux event. These methods lead to the most reliable symptom recorded albeit consuming time. However, the small number and inhomogenous characteristic of the subjects are the main limitation of this pilot study. Further large study that highlights the accurate symptom association using real-time video or developed artificial intelligence MII-pH machine for children suspected of GERD should be more meritorious.

ARTICLE HIGHLIGHTS

Research background

Esophageal atresia (EA) is a common digestive malformation with increasing risk of esophageal complications even after successful surgical correction. Gastroesophageal reflux disease (GERD) is the frequent gastrointestinal co-morbidity causing serious long-term consequences namely esophageal stricture and esophageal carcinoma. Hence, early detection and prompt treatment are crucial.

Research motivation

This research aimed to study the prevalence of GERD using esophageal histopathology and the novel tool, combined Video Multichannel Intraluminal Impedance and pH (MII-pH) study, in children diagnosed with EA. We believe that symptoms from video monitoring should be recorded more precisely with time reflux than by caregivers and make the interpretation of reflux-symptom association more meaningful.

Research objectives

To investigate the prevalence of GERD and the symptom association in children diagnosed EA by combined Video MII-pH study.

Research methods

Seventeen investigations that included esophagogastroduodenoscopy with biopsy and combined Video MII-pH study were performed in 15 children diagnosed EA. All signs and symptoms of GERD from video were recorded during MII-pH monitoring. MII-pH study was manually analysis including the symptom-reflux association using the symptoms from video record. Diagnostic value of combined Video MII-pH study was calculated using the result of esophageal histopathology as the gold standard to diagnose GERD.

Research results

The total symptoms recorded from video of all 17 combined Video MII-pH monitoring were cough (67.3%), vomiting (17.3%), irritability or unexplained crying (13.4%) and dysphagia (1.9%). In aspect of symptom association, vomiting was the symptom that mostly associated with reflux followed by irritability or unexplained crying and cough. Using esophageal histopathology as the gold standard for GERD, combined Video MII-pH has high diagnostic value with the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of 72.7%, 100%, 100%, 67% and 82.4%, respectively.

Research conclusions

Prevalence of GERD in children with EA was high. Combined Video MII-pH study to detect GERD in children with EA had high diagnostic value with the trend of specific symptom association.

Research perspectives

II-pH study has limitation to be the gold-standard test for GERD as the data of the reflux value in each age group are scarce. To improve the utility and diagnostic value of this machine, we synchronized the video recording during the study. The precise time of symptom recorded from video could increase symptom-reflux association albeit its time consuming. In the future, real-time Video MII-pH monitoring machine should be developed to improve the accuracy and clinical utility of MII-pH study.

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Randomized Controlled Trial

Epigastric pain syndrome: What can traditional Chinese medicine do? A randomized controlled trial of Biling Weitong Granules

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Abstract

BACKGROUND

Recent research suggests that although prokinetic agents, acid suppressors, and radical treatment for *Helicobacter pylori* infection may be effective in patients with functional dyspepsia (FD), a large proportion of patients still fail to respond to these treatments or may suffer from severe adverse reactions. Many traditional

Institutional review board

statement: This study was approved by the IRB of Xiyuan Hospital of China Academy of Chinese Medical Sciences (No. 2016XL011).

Clinical trial registration statement:

This study is registered at <https://http://www.chictr.org.cn/showproj.aspx?proj=18562>. The registration identification number is ChiCTR17010953./

Informed consent statement:

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

Conflict-of-interest statement:

We declare that we have no potential conflicting interests related to this paper.

Data sharing statement:

No additional data are available.

CONSORT 2010 statement:

The authors have read the CONSORT 2010 Statement—checklist of items, and the manuscript was prepared and revised according to the CONSORT 2010 Statement—checklist of items.

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Chinese medicinal herbs can regulate the status of the entire body and have special advantages in the treatment of functional diseases. The present study was designed to verify the efficacy of Biling Weitong Granules (BLWTG), a traditional Chinese medicinal herbal compound formula, in alleviating epigastric pain syndrome (EPS) in FD patients, in an attempt to provide an effective prescription for the clinical treatment of this disease.

AIM

To evaluate the clinical efficacy and safety of BLWTG in treating EPS in patients with FD.

METHODS

In this multicenter, stratified, randomized, double-blind, placebo-controlled, parallel group clinical trial, eligible patients were randomized into the BLWTG and placebo groups who were treated for 6 wk. Efficacy indicators including the severity and frequency of EPS and the time to pain resolution and safety indicators including adverse events were observed and compared.

RESULTS

The baseline demographic data and clinical characteristics, such as epigastric pain symptoms, pain intensity, and frequency of attacks, were matched between the two groups before randomization. After 6 wk of treatment and after the center effect was eliminated, the epigastric pain was significantly improved in 28.33% and 85.59% of the patients in the placebo and BLWTG groups, respectively ($P < 0.05$). At 6 wk, the resolution rate of epigastric pain was 15% and 69.49% in the placebo and BLWTG groups, respectively ($P < 0.05$). The differences of total FD clinical score between these two groups were significant ($P < 0.05$) at 2, 4, and 6 wk ($P < 0.05$). The scores of each item and the total score in the Functional Digestive Disorders Quality of Life Questionnaire showed significant differences between the two groups at 6 wk after both the center and interaction effects were eliminated ($P < 0.05$). There was no significant difference in the incidence of adverse events between the two groups, and no serious adverse event was noted during the observation.

CONCLUSION

Compared with placebo, BLWTG markedly improved EPS in FD patients without causing serious adverse reactions.

Key words: Biling Weitong Granules; Compound formula; Traditional Chinese medicine; Functional dyspepsia; Epigastric pain syndrome; Randomized controlled trial

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Core tip: Although the currently available drugs for functional dyspepsia (FD) can, to some extent, improve the symptoms, they are still ineffective or have severe adverse reactions in some patients. The present study evaluated the clinical efficacy and safety of Biling Weitong Granules in treating epigastric pain syndrome in FD patients. Compared with placebo, Biling Weitong Granules markedly relieved the epigastric pain syndrome symptoms and significantly improved the total FD clinical score based on symptoms including postprandial fullness and discomfort, early satiety, epigastric pain, epigastric burning, belching, and pharyngeal obstruction, decreased appetite, fatigue, limb weakness, and irritability, thus, it improved the quality of life without causing serious adverse reactions.

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INTRODUCTION

Functional dyspepsia (FD) is a functional gastric disorder presenting with dyspeptic symptoms such as gastric pain, postprandial fullness, and early satiety. The new Rome IV diagnostic criteria for FD (2016) divides FD into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS), based on the main symptoms and possible mechanisms of this disease^[1]. Although the pathogenesis of FD remains unknown, it is currently believed that both PDS and EPS may be related to a variety of factors such as gastroduodenal dysfunction, visceral hypersensitivity, *Helicobacter pylori* (*H. pylori*) infection, and mental stress^[2]. However, more studies are required to verify these findings. Prokinetic drugs are effective in some FD patients. Many randomized controlled clinical trials have shown that prokinetic drugs such as cisapride in FD patients have a significantly higher response rate than placebo, because they markedly alleviate symptoms such as dyspepsia and epigastric fullness; however, the cardiac toxicity of these synthetic drugs limits their clinical application^[3,4]. Acid-suppressive drugs can reduce the stimulation of gastric mucosa by gastric acid and may improve abdominal pain in FD patients, although they cannot alleviate all the symptoms^[5].

Biling Weitong Granules (BLWTG) are a newly developed traditional Chinese medicine (TCM) formula based on the ancient formulas Jinlingzi San and Zuojin Wan, with main ingredients including *Fructus Litseae*, *Fructus Meliae Toosendan*, *Rhizoma Corydalis*, *Radix et Rhizoma Rhei*, *Rhizoma Coptidis*, *Fructus Evodiae*, *Rhizoma Cyperi*, *Fructus Citri*, *Fructus Citri Sarcodactylis*, *Endoconcha Sepia*, and *Concha Arcae*. An animal experiment showed that BLWTG can inhibit gastric acid secretion and lower total acidity, thus alleviating gastric pain^[6]. Existing randomized controlled trials have shown that BLWTG has a certain therapeutic effect on gastric pain, heartburn, acid reflux, and other symptoms associated with chronic gastritis and peptic ulcers^[7,8]. Although BLWTG has been clinically applied for the treatment of EPS in FD patients, there is still no evidence from well-designed, large, multicenter, placebo-controlled, randomized double-blind clinical trials.

MATERIALS AND METHODS

Diagnostic criteria

Western medicine-based diagnostic criteria: The diagnosis was based on the New Rome IV Criteria for Functional Gastrointestinal Disorders. One or more of the following criteria had to be met before a diagnosis of FD was made: (1) Postprandial fullness; (2) Early satiety; (3) Epigastric pain; and (4) Epigastric burning; and there was no evidence of organic disease (including at upper endoscopy) that was likely to explain the symptoms. The criteria were fulfilled for the last 3 mo with symptom onset at least 6 mo before diagnosis^[1].

TCM-based diagnostic criteria: Syndrome of stagnation of liver qi. Primary symptoms: (1) Migratory epigastric and hypochondriac distending pain; (2) Epigastric distress and belching; (3) Impatience and agitation; and (4) Wiry pulse. Secondary symptoms: (1) Bitter taste in mouth; (2) Depression and frequent sighing; (3) Sensation of foreign body in throat; (4) Heartburn or acid regurgitation; (5) Abdominal distention and anorexia/vomiting; and (6) Pink tongue or red tongue tip/borders, along with thin and yellow fur.

Syndrome of liver qi invading the stomach. Primary symptoms: (1) Epigastric distention and fullness, affecting both hypochondria, which can be induced or worsened during emotional frustration; (2) Belching and hiccups; (3) Heartburn or acid regurgitation; (4) Impatience and agitation; and (5) Small and wiry pulse. Minor symptoms: (1) Walking qi and scurrying pain in both hypochondria; (2) Dry mouth and bitter taste in mouth; (3) Deep-colored urine; and (4) Dull and red tongue quality and thin (or thick) and whitish fur.

Syndrome of deficiency of spleen qi and stomach qi. Primary symptoms: (1) Abdominal distention, fullness, and pain, and the pain became worse after exertion or when hungry; (2) Poor appetite; (3) Loose stools; and (4) Pale and plump tongue with tooth marks, along with thin white or greasy fur. Minor symptoms: (1) Vomiting of clear fluid; (2) Belching and irritation; (3) Tastelessness without thirstiness; (4) Dizziness and fatigue; and (5) Small and weak pulse.

Syndrome of dampness-heat accumulated in stomach. Primary symptoms: (1) Gastric distention, fullness, and discomfort; (2) Nausea or vomiting; (3) Poor appetite; (4) Belching and irritation; and (5) Red tongue and yellow greasy fur. Minor

symptoms: (1) Heavy sensation of head and body and weak limbs; (2) Bitter taste in mouth and acid regurgitation; (3) Difficulty in defecation; (4) Dark-colored urine; and (5) Soft/small and rapid pulse.

Syndrome of intermingled heat and cold. Primary symptoms: (1) Gastric distention, fullness, or pain; (2) Gastric upset or discomfort; (3) Irritation, dry mouth, and bitter taste in mouth; and (4) Abdominal fullness and borborygmus, which were worsened in cold days. Minor symptoms: (1) Abdominal coldness; (2) Belching and poor appetite; (3) Occasional dark-colored urine; (4) Pale tongue with yellow fur; and (5) Small (or smooth) and wiry pulse. A TCM-based syndrome was identified if two primary symptoms plus one minor symptom were detected or if one primary symptom plus two minor symptoms were detected^[9].

Inclusion criteria

(1) Outpatients aged 18–years-old to 65-years-old; (2) Meeting the diagnostic criteria for EPS in the New Rome IV Criteria for Functional Gastrointestinal Disorders; (3) Epigastric pain; and (4) Karnofsky performance status score ≥ 4 .

Exclusion criteria

(1) Abnormal findings on hepatobiliary ultrasound, gastroscopy, and/or laboratory tests; (2) Evidence of gastrointestinal bleeding and/or inflammation (ulcers and erosions), including black stools and hematemesis; (3) Palpable abdominal masses; (4) Positive result for *H. pylori* test; (5) Progressive dysphagia and swallowing pain, persistent vomiting, and/or unconscious weight loss; (6) History of gastric surgery; (7) Immune disorder (*e.g.*, leukemia and tumor) or use of an immunoinhibitor or glucocorticoids within the past 3 mo; (8) Severe cardiac insufficiency, liver and kidney dysfunction (alanine aminotransferase and aspartate aminotransferase levels were 1.5 times upper limit of normal), endocrine disorder, and/or hematopoietic disorder; or, hematological tests revealed iron deficiency anemia; (9) Mental illness, intellectual disability, and/or language impairments; (10) Pregnancy (positive pregnancy test for women of childbearing age) or lactation; (11) Allergy to the components of this drug; (12) Participation in a clinical trial within the past 3 mo; (13) Confirmed or presumed alcoholism and/or drug abuse, or current use of anxiolytic, antidepressant, or insomnia drugs; and (14) Individuals who were regarded as not feasible for this clinical trial.

Interventions

BLWTG: BLWTG (Jiangsu Pharmaceutical Co., Ltd., Yangzijiang Pharmaceutical Group; batch number: Z19990069) was composed of the following TCM crude drugs: *Fructus Litseae* (2.515 g/pack), *Fructus Meliae Toosendan* (2.515 g/pack), *Rhizoma Corydalis* (1.510 g/pack), *Radix et Rhizoma Rhei* (0.755 g/pack), *Rhizoma Coptidis* (0.755 g/pack), *Fructus Evodiae* (0.380 g/pack), *Rhizoma Cyperi* (2.515 g/pack), *Fructus Citri* (2.515 g/pack), *Fructus Citri Sarcodactylis* (1.510 g/pack), *Endoconcha Sepia* (2.515 g/pack), and *Concha Arcae* (2.515 g/pack). Each pack of BLWTG weighed 5 g, equal to 20 g of crude drugs. Quality control of the effective components including berberine hydrochloride, tetrahydropalmatine, chlorogenic acid, and emodin showed stable results in three batches of BLWTG [Appendix 1 (Supplementary Table 1) shows the drug components and their crude drug contents and Appendix 2 (Supplementary Tables 2–7 and Figure 1) shows the compound composition and fingerprint profile of the drugs]. According to the TCM principle, BLWTG is effective in activating qi for flowing stagnation, promoting blood for alleviating pain, dispersing stagnated liver qi for relieving qi stagnation, and harmonizing stomach for suppressing acid reflux. It is particularly useful for patients with epigastric pain caused by qi stagnation and blood stasis. The indications of this proprietary Chinese medicine in this trial were the same as those published.

Placebo: The placebo (5 g per pack; provided by Jiangsu Pharmaceutical Co., Ltd., Yangzijiang Pharmaceutical Group) did not contain any drug, and its adjuvants (dextrin, stevia, and low-substituted hydroxypropyl cellulose) were the same as for BLWTG. Also, the placebo had basically the same taste, odor, and color as BLWTG, and the use of colorants met the requirements in the Quality Standards for Pharmaceutical Excipients released by the Chinese authority. The quality standards and testing methods of the placebo were consistent with BLWTG, and the results met the proposed quality standards. The simulated effect of the placebo was evaluated before the initiation of the clinical trial, so as to determine its consistency with BLWT [Appendix 3 (Supplementary Tables 8–10 and Figure 2)].

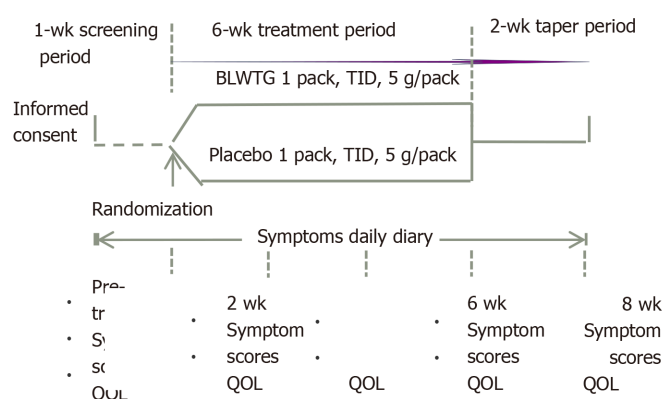


Figure 1 Study design. BLWTG: Biling Weitong Granules; QOL: Quality of life.

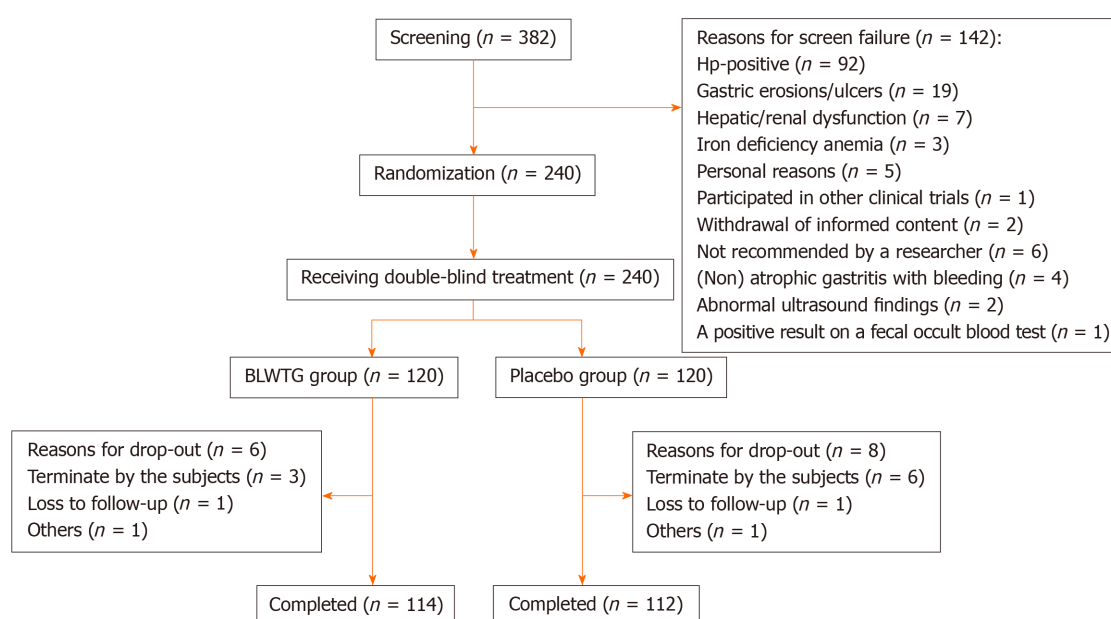


Figure 2 Flowchart for subject enrollment, randomization, and follow-up.

Aluminum hydroxide/ magnesium hydroxide tablets: Aluminum hydroxide/ magnesium hydroxide tablets (trade name: Talcid, Bayer HealthCare Co., Ltd.), 0.5 g, was used with the permission of the research physicians.

Route of administration: Both BLWTG and placebo were orally administered with warm water at a dose of 5 g tid for 6 consecutive weeks. When pain was unbearable in the placebo group, the patient contacted the research physician by telephone, and the physician asked the patient about the pain and decided whether Talcid should be given. Talcid (one or two tablets) was orally administered by chewing tid 1–2 h after a meal, before bedtime, or when stomach discomfort occurred.

Study design

In this multicenter, randomized, double-blind, placebo-controlled, parallel group study, patients were recruited from eight hospitals and then randomized into the BLWTG and placebo groups in a 1:1 ratio. The study was divided into three stages: a 1-wk screening period, a 6-wk treatment period, and a 2-wk taper period. Patients were evaluated during the screening period, 2, 4 and 6 wk after treatment, and 2 wk after drug withdrawal. Patients in the BLWTG group were treated with oral BLWTG 5 g tid with warm water. The placebo group was treated with placebo in the same way as the BLWTG group. During the trial, no additional TCM or western drugs related to the

treatment of this disease were allowed. Also, no TCM such as acupuncture, massage, and cupping that may affect the treatment response could be used. During the medication period, if the epigastric pain had not been reported for ≥ 2 wk, it was judged that the pain had disappeared. Medication was stopped, and the patient received all the relevant examinations before exiting from the study. Such a case was defined as a clinically cured case. During the medication period, if a patient had consumed 20 or more Talcid tablets, they immediately contacted their research physician to terminate the treatment. The patient received all the relevant examinations before exiting from the study. Such a patient was defined as a clinically unresponsive case. **Figure 1** is the flowchart of this study.

The trial was approved by the ethics committees of all participating hospitals, and all the patients gave signed informed consent. This trial is registered at <http://www.chictr.org.cn> (registration number: ChiCTRIPR17010953).

Randomization and blinding

Based on the principle of stratified blocked randomization, random numbers were generated using SAS 9.3 statistical software to number the drugs before packaging. The drugs were used sequentially according to the screening order of the patients.

Prior to drug blinding, we evaluated whether placebo was similar to BLWTG in terms of shape, texture, color, odor, and taste. For studies performed in patients with gastrointestinal diseases, a qualified placebo must have basically the same shape, taste, odor, and color as the test drug, so that the subjects cannot distinguish between these two products. In the present study, patients could not distinguish placebo from BLWTG by shape, texture, color, odor, or taste, suggesting the placebo met the requirements for blinding. The evaluation results are shown in Appendix 3. The blind codes were divided into classes I and II. Class I blind code indicated whether a specific drug belonged to drug A or B, whereas class II blind code indicated whether drug A or B belonged to BLWTG or placebo. The class I and class II blind codes were stored by the research hospitals and sponsor, respectively.

Outcome measurements

Primary endpoints: Improvement of epigastric pain after 6 wk of medication was the primary endpoint. Decrease in the total score of the degree and frequency of epigastric pain at 6 wk after medication by $\geq 50\%$ from baseline was defined as clinically effective. The visual analog score (VAS) was used to evaluate pain severity. The VAS comprised 10-cm lines that marked at the extremes no pain and worst pain imaginable. Patients recorded the levels of pain intensity on the lines. The patients recorded their highest VAS value of epigastric pain in their diary cards, and the researchers used the highest VAS score in the diary cards as the score of the week for epigastric pain. There was one VAS score every week. The number of days of epigastric pain was recorded every week, and the number of days of epigastric pain during the treatment period was analyzed. Total score for severity and frequency of epigastric pain was based on the following criteria: 0 for both severity and frequency if there were no pain; $0 < \text{VAS} < 4$, (1) For severity; $4 \leq \text{VAS} < 7$, (2) For severity; and $7 \leq \text{VAS} \leq 10$, and (3) For severity; (1) For frequency for pain onset ≤ 1 d/wk; (2) For frequency for pain onset 2–4 d/wk; and (3) For frequency for pain onset ≥ 5 d/wk. The total weekly score was the sum of the severity score and frequency score, and the severity was scored based on the maximum VAS score within a week and evaluated once weekly.

Secondary endpoints: Pain resolution was defined as no epigastric pain within the past ≥ 2 wk. FD was scored based on symptoms including postprandial fullness and discomfort, early satiety, epigastric pain, epigastric burning, belching, pharyngeal obstruction, decreased appetite, fatigue, limb weakness, and irritability. 0 represented no such symptoms. The severity of a symptom was divided into three grades: 3, grade I (mild symptom); 5, grade II (moderate symptom); and 7, grade III (severe symptom). The total score of these symptoms was calculated and evaluated at day 0, week 2, week 4, and week 6. The Functional Digestive Disorders Quality of Life Questionnaire (FDDQL) contained 43 items in eight domains, namely daily activities (Q1–Q8), anxiety (Q9–Q13), diet (Q14–Q19), sleep (Q20–Q22), discomfort (Q23–Q31), coping with disease (Q32–Q37), control of disease (Q38–Q40), and stress (Q41–Q43). FDDQL was originally developed in French and its Chinese version has been validated in terms of reliability, validity, and responsiveness^[10]. The FDDQL scores were calculated before treatment and 2, 4 and 6 wk after treatment and compared between the two groups. The use of Talcid tablets during the observation period was evaluated.

Compliance and safety evaluation

Medication compliance was evaluated based on the ratio between actual dose and desired dose. It was regarded as good if the ratio ranged between 80% and 120%. Difference in medication compliance was compared between these two groups 6 wk after treatment. The changes in laboratory test results, electrocardiographic findings, and vital signs before and after drug administration, and the adverse events and their incidences during the clinical research were also compared.

Statistical analysis

According to the literature^[7,8,11-14], the clinical response rate of BLWT in treating EPS in FD patients was 70% [50% calculated, with 95% confidence interval (CI) ($\alpha = 0.05$) and 80% power ($\beta = 0.2$)]. The ratio between BLWTG and placebo was designed to be 1:1, and the sample size was estimated. There were 94 cases in the BLWTG and 94 cases in the placebo groups. The quality of the study was strictly controlled during the observation, and the rate of loss to follow-up was controlled within 20%. The expected total number of cases was 240, with 120 in each group. Statistical analysis was performed using SAS 9.3 software. Two-sided tests were used for all analyses. $P \leq 0.05$ was regarded as significant unless otherwise indicated. A 95%CI was used. Based on the intention-to-treat principle, the last observation carried forward method was applied for imputing missing data (*i.e.* the missing efficacy data was replaced by the efficacy data of the last follow-up). The efficacy analysis was mainly based on a full analysis set (FAS), and the central effect was considered. The effectiveness in these two groups was compared using the Cochran-Mantel-Haenszel- χ^2 test with and without center stratification, and the 95%CI of the difference between two groups was calculated. Comparisons of epigastric pain resolution were based on χ^2 test or Fisher's exact test. The safety analysis was mainly based on the descriptive statistics. The adverse events that occurred in this trial are described in a list and their incidences were compared using Fisher's exact probability test.

RESULTS

Study population

A total of 382 patients were screened, and 142 were ruled out in accordance with the inclusion/exclusion criteria. Of 240 patients who were randomly enrolled, two were excluded due to lack of medication record and did not enter the FAS; 14 failed to enter the per protocol analysis due to reasons such as loss to follow-up, withdrawal from study, and low medication compliance. The drop-out rate showed no significant difference between these two groups ($P > 0.05$). Patient enrolment, randomization and follow-up are shown in Figure 2.

The demographic characteristics (age, gender, height, weight, and education level) showed no significant difference between these two groups in FAS analysis (all $P > 0.05$) (Table 1).

Baseline analysis of efficacy indicators

The two groups also showed no significant differences in total epigastric pain score, epigastric pain frequency, total FD clinical score, and FDDQL score, suggesting the disease conditions were comparable between these two groups before treatment (Table 2).

Primary endpoints: The clinical response rate for epigastric pain was 85.59% in the BLWT group and 28.33% in the placebo group ($P < 0.05$, after the center effect was eliminated) (Table 3).

Secondary endpoints: The epigastric pain resolution rate was 69.49% in the BLWT group and 15% in the placebo group ($P < 0.0001$, after the center effect was eliminated) (Table 4).

Change in total epigastric pain score (severity score + frequency score) over time was measured on a weekly basis, and the baseline values were matched between the two groups ($P = 0.8748$). In contrast, the difference became significant 1 wk after treatment ($P = 0.0125$) and the score remained significantly lower in the BLWTG group than in the placebo group 2 wk after drug discontinuation ($P < 0.0001$) (Table 5).

The total FD clinical score based on symptoms including postprandial fullness and discomfort, early satiety, epigastric pain, epigastric burning, belching, pharyngeal obstruction, decreased appetite, fatigue, limb weakness, and irritability was not

Table 1 Baseline characteristics of study population in a full analysis set

	Placebo group, <i>n</i> = 120	BLWT group, <i>n</i> = 118	<i>P</i> value
Sex, <i>n</i>			0.8656
Male/female	45/75	43/75	
Age in yr, mean \pm SD	37.78 \pm 13.96	37.95 \pm 13.38	0.9256
Body height in cm, mean \pm SD	166.69 \pm 7.98	165.46 \pm 7.33	0.214
Body weight in kg, mean \pm SD	62.93 \pm 13.05	61.22 \pm 11.06	0.2765
Education background, <i>n</i> (%)			0.9256
Illiteracy	1 (0.83)	1 (0.85)	
Primary school	5 (4.17)	4 (3.39)	
Middle school	19 (15.83)	25 (21.19)	
High school	19 (15.83)	18 (15.25)	
Junior college	16 (13.33)	14 (11.86)	
College	16 (13.33)	19 (16.1)	
Higher than college	44 (36.67)	37 (31.36)	

BLWTG: Biling Weitong Granules.

Table 2 Baseline disease conditions in two groups, mean \pm SD

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Total epigastric pain score	4.63 \pm 0.73	4.61 \pm 0.72	0.8748
VAS score of the severity of epigastric pain	6.17 \pm 1.14	6.25 \pm 1.2	0.6257
Frequency of epigastric pain, d/wk	4.14 \pm 1.73	4.08 \pm 1.67	0.7964
Total FD clinical score	28.18 \pm 9.02	29.07 \pm 9.33	0.458
FDDQL score	58.91 \pm 13.78	57.15 \pm 15.35	0.3518

BLWTG: Biling Weitong Granules; FD: Functional dyspepsia; FDDQL: Functional Digestive Disorders Quality of Life Questionnaire; VAS: Visual analog score.

significantly different between the two groups at baseline ($P = 0.458$). It became significantly lower in the BLWT group than placebo group at weeks 2, 4 and 6 ($P < 0.05$), (Table 6).

The total FDDQL score and the scores of its six dimensions including daily activities, anxiety, diet, sleep, discomfort, coping with disease, control of disease, and stress showed significant differences between the BLWTG and placebo groups at week 6 ($P < 0.05$, after the center effect and interaction effect were eliminated). The increases in total FDDQL score and the score of each dimension were significantly higher than in the placebo group (Table 7).

The rate of Talcid use during the observation period was 7.63% in the BLWTG group and 24.37% in the placebo group ($P < 0.05$, after the center effect was eliminated) (Table 8).

Adverse events

A total of 41 adverse events occurred in 28 patients during the trial. Fifteen patients (12.5%) in the BLWTG group experienced 23 adverse events, of which one (0.83%) was an adverse reaction (diarrhea) and no serious adverse event was noted. Thirteen patients (10.93%) in the placebo group experienced 18 adverse events. The incidence of adverse events was not significantly different between these two groups ($P > 0.05$).

Table 3 Analysis of clinical response for epigastric pain in a full analysis set, *n* (%)

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Total			< 0.0001
Responsive	34 (28.33)	101 (85.59)	
Not responsive	86 (71.67)	17 (14.41)	
Baseline VAS score, 4 ≤ VAS < 7			
Responsive	27 (30.34)	77 (88.51)	< 0.0001
Not responsive	62 (69.66)	10 (11.49)	
Baseline VAS score, 7 ≤ VAS ≤ 10			
Responsive	7 (22.58)	24 (77.42)	< 0.0001
Not responsive	24 (77.42)	7 (22.58)	

BLWTG: Biling Weitong Granules; VAS: Visual analog score.

Table 4 Rate of abdominal pain resolution in two groups, *n* (%)

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Resolution rate			< 0.0001
Resolved	18 (15)	82 (69.49)	
Not resolved	102 (85)	36 (30.51)	

BLWTG: Biling Weitong Granules.

Table 5 Total epigastric pain score (mean ± SD) in functional dyspepsia patients in a full analysis set

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Baseline	4.63 ± 0.73	4.61 ± 0.72	0.8748
Week 1	4.43 ± 1.17	4.03 ± 1.28	0.0125
Week 2	3.65 ± 1.63	3.2 ± 1.59	0.0335
Week 3	3.67 ± 1.42	2.81 ± 1.46	< 0.0001
Week 4	3.45 ± 1.48	2.38 ± 1.7	< 0.0001
Week 5	3.23 ± 1.75	1.84 ± 1.71	< 0.0001
Week 6	3.19 ± 1.56	1.32 ± 1.5	< 0.0001

BLWTG: Biling Weitong Granules.

DISCUSSION

The recurrent epigastric pain in FD patients seriously affects quality of life, leading to frequent treatment-seeking behaviors and huge demand for medical resources. The treatments commonly used in western medicine include acid inhibitors, gastric mucosal protective agents, and gastrointestinal stimulants but their effectiveness remains unsatisfactory. A variety of TCM-based therapies has been developed for FD. For instance, acupuncture has been applied in the clinical treatment of FD^[15]; topical application of drugs has been used in patients with gastric pain belonging to the Type of Deficiency-cold of Spleen and Stomach^[16]; and massage has also been applied^[17]. The effectiveness and safety of TCM medications for FD have been validated. For example, Xiangsha Liujunzi Granules significantly improved early satiety and PDS in FD patients^[18]; administration of Zhizhu Kuanzhong capsules was piementerior over placebo in treating PDS, with a response rate of up to 54.7%^[6]. However, few studies

Table 6 Total functional dyspepsia clinical score (mean \pm SD) in two groups in a full analysis set

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Baseline	28.18 \pm 9.02	29.07 \pm 9.33	0.458
Week 2	23.8 \pm 9.15	20.53 \pm 9.77	0.0085
Week 4	20.69 \pm 9.96	14.12 \pm 8.45	< 0.0001
Week 6	20.52 \pm 9.31	7.76 \pm 6.67	< 0.0001

BLWTG: Biling Weitong Granules.

Table 7 Functional Digestive Disorders Quality of Life Questionnaire total score and scores of different dimensions (6-wk baseline) (mean \pm SD) in a full analysis set

	Placebo group, <i>n</i> = 120	BLWTG group, <i>n</i> = 118	<i>P</i> value
Total score	3.93 \pm 14.78	20.14 \pm 15.7	< 0.0001
Daily activities	4.72 \pm 18.61	19.58 \pm 19.16	< 0.0001
Anxiety	6.42 \pm 22.51	25.24 \pm 21.49	< 0.0001
Diet	11.77 \pm 74.07	20.51 \pm 20.59	0.2498
Sleep	1.06 \pm 18.34	19.1 \pm 20.27	< 0.0001
Discomfort	4.86 \pm 17.34	21.46 \pm 18.15	< 0.0001
Coping with disease	0.45 \pm 17.28	15.13 \pm 18.72	< 0.0001
Control of disease	6.45 \pm 23.65	23.62 \pm 25.25	< 0.0001
Stress	7.03 \pm 23.5	18.77 \pm 22.52	0.0003

BLWTG: Biling Weitong Granules.

Table 8 Use of Talcid tablets during the observation period in a full analysis set

	Placebo group	BLWTG group	<i>P</i> value
Use rate, <i>n</i> (%)			
Number of users	29 (24.37)	9 (7.63)	0.0003
Number of non-users	90 (75.63)	109 (92.37)	

BLWTG: Biling Weitong Granules.

have explored the role of TCM medications for EPS in FD patients.

Based on the ancient formulas Jinlingzi San and Zuojin Wan, BLWTG was developed by Dr. Dong Jianhua (1918–2001), with its main ingredients including *Fructus Litseae*, *Fructus Meliae*, *Toosendan*, *Rhizoma Corydalis*, *Radix et Rhizoma Rhei*, *Rhizoma Coptidis*, *Fructus Evodiae*, *Rhizoma Cyperi*, *Fructus Citri*, *Fructus Citri Sarcodactylis*, *Endoconcha Sepia*, and *Concha Arcae*. BLWTG was licensed in China in 2016 and is currently widely used in clinical practice. Its annual sales reached 350 million RMB yuan in 2019. Pharmacodynamic studies have found that BLWTG can inhibit gastric acid secretion, increase the pH level of gastric juice, and suppress pepsin activity; it can prevent and repair gastric mucosal damage and have analgesic and antispasmodic effects on gastric pain spasm. Based on the drug formula, the drug contents and pharmacological studies on BLWTG as shown in [Supplementary Table 1](#) (Appendix I) may help to reveal the mechanism of action of this formula in treating EPS in FD patients. The present study demonstrated the excellent efficacy of BLWTG in alleviating pain. After 6 wk of treatment, the response rate and epigastric pain resolution rate were significantly higher in the BLWTG group than placebo group (clinical response rate: 85.59% *vs* 28.33%; pain resolution rate: 69.49% *vs* 15%), which is

consistent with the TCM theory, findings of modern pharmacological research, and clinical experiences related to the composition of the formula. Thus, BLWTG may be a good analgesic option for EPS in FD patients.

However, there were some limitations in this study. First, this study only included FD patients with EPS and the results might not be applicable for patients with upper gastrointestinal pain due to other upper abdominal diseases. Second, our study did not include *H. pylori*-positive patients, which also limited the extrapolation of BLWTG to other patient populations. Thirdly, parallel controlled studies comparing BLWTG and PPI may be carried out in future to identify the patients who will benefit most from BLWTG and set the precise dose ranges. Finally, BLWTG is composed of 11 TCM ingredients and its complex action mechanisms warrant further investigation.

ARTICLE HIGHLIGHTS

Research background

Recent research has suggested that although prokinetic agents, acid suppressors, and radical treatment for *Helicobacter pylori* infection may be effective in patients with functional dyspepsia (FD), a large proportion of patients still failed to respond to these treatments or may suffer from severe adverse reactions. Many traditional Chinese medicinal (TCM) herbs can regulate the status of entire body and have shown special advantages in the treatment of functional diseases.

Research motivation

Although the currently available drugs for FD can, to some extent, improve the symptoms, they are still ineffective or have severe adverse reactions in some patients. The present study was designed to verify the efficacy of Biling Weitong Granules (BLWTG), a TCM herbal compound formula, in alleviating the epigastric pain syndrome (EPS) in FD patients, in an attempt to provide an effective prescription for the clinical treatment of this disease.

Research objectives

The aim of the study was to evaluate the clinical efficacy and safety of BLWTG in treating EPS in patients with FD.

Research methods

In this multicenter, stratified, randomized, double-blind, placebo-controlled, parallel group clinical trial, eligible patients were randomized into the BLWTG and placebo groups who were treated for 6 wk. Efficacy indicators including the severity and frequency of EPS, the resolution rate of epigastric pain, the total score of FD symptoms and the Functional Digestive Disorders Quality of Life Questionnaire scores and safety indicators including adverse events were observed and compared. Two-sided tests were used for all analyses. $P \leq 0.05$ was regarded as significant unless otherwise indicated. A 95% confidence interval was used. Based on the intention-to-treat principle, the last observation carried forward method was applied for imputing missing data (*i.e.* the missing efficacy data was replaced by the efficacy data of the last follow-up). The efficacy analysis was mainly based on a full analysis set, and the central effect was considered.

Research results

The baseline demographic data and clinical characteristics, such as epigastric pain symptoms, pain intensity, and frequency of attacks, were matched between the two groups before randomization. After 6 wk of treatment and after the center effect was eliminated, the epigastric pain was significantly improved in 28.33% and 85.59% of the patients in the placebo and BLWTG groups, respectively ($P < 0.05$). At 6 wk, the resolution rate of epigastric pain was 15% and 69.49% in the placebo and BLWTG groups, respectively ($P < 0.05$). The differences of total FD clinical score between these two groups were significant ($P < 0.05$) at 2, 4 and 6 wk ($P < 0.05$). The scores of each item and the total score in the Functional Digestive Disorders Quality of Life Questionnaire showed significant differences between the two groups at 6 wk after both the center and interaction effects were eliminated ($P < 0.05$). There was no significant difference in the incidence of adverse events between the two groups, and no serious adverse event was noted during the observation.

Research conclusions

Compared with placebo, BLWTG markedly improved EPS in FD patients without causing serious adverse reactions. BLWTG may be a good analgesic option for EPS in FD patients.

Research perspectives

This study only included FD patients with EPS and the results might not be applicable for patients with upper gastrointestinal pain due to other upper abdominal diseases. Our study did not include *Helicobacter pylori*-positive patients, which also limited the extrapolation of BLWTG to other patient populations. BLWTG is composed of 11 TCM ingredients and its complex action mechanisms warrant further investigation.

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