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EDITORIAL

Role of cancer stem cells in age-related rise in colorectal cancer

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Author contributions: Nangia-Makker P designed and wrote the editorial; Yu Y designed and performed the experiments; Majumdar APN was invited to contribute to the editorial, conceptualized and wrote the editorial, and contributed to research design.

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Abstract

Colorectal cancer (CRC) that comprises about 50% of estimated gastrointestinal cancers remains a high mortality malignancy. It is estimated that CRC will result in 9% of all cancer related deaths. CRC is the third leading malignancy affecting both males and females equally; with 9% of the estimated new cancer cases and 9% cancer related deaths. Sporadic CRC, whose incidence increases markedly with advancing age, occurs in 80%-85% patients diagnosed with CRC. Little is known about the precise biochemical mechanisms responsible for the rise in CRC with aging. However, many probable reasons for this increase have been suggested; among others they include altered carcinogen metabolism and the cumulative effects of long-term exposure to cancer-causing agents. Herein, we propose a role for self-renewing, cancer stem cells (CSCs) in regulating these cellular events. In this editorial, we have briefly described the recent work on the evolution of CSCs in gastro-intestinal track especially in the colon, and how they are involved in the age-related rise in CRC. Focus of this editorial is to provide a description of (1) CSC; (2) epigenetic and genetic mechanisms giving rise to CSCs; (3) markers of CSC; (4) characteristics; and (5) age-related increase in CSC in the colonic crypt.

Key words: Cancer stem cells; Aging; Colorectal cancer; Colonospheres; Colonic crypt

Core tip: Sporadic colorectal cancer (CRC), an age-related disease, occurs in 80%-85% of patients with CRC. The changes that occur at the cellular and molecular levels during ageing leading to a rise in CRC are poorly understood. We have postulated a role for cancer stem/stem-like cells that are shown to possess self-renewing, pluripotent properties. These cells, which reside at the bottom of the colonic crypt, are thought to regulate the processes of carcinogenesis. In this

editorial, we have briefly described the recent work on the evolution of cancer stem cells in gastro-intestinal tract with particular reference to the colon, and how they are involved in the development and progression of CRC, the incidence of which increases with advancing age.

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TEXT

The primary challenge in the study of aging is to gain an in-depth understanding of the intricate relationship between disease processes and aging. One of the most consistent pathological conditions with advancing age is a sharp rise in colorectal cancer (CRC), which typically occurs after the age of 50. It has been reported that a male in age group 70 years and above exhibits 54 times greater risk of developing CRC compared to younger male (birth to 39 years).

According to a well accepted model of CRC progression by Vogelstein, this malignancy arises as a result of accumulation of mutations in tumor suppressor genes and oncogenes[1,2]. For a malignant tumor to be initiated mutations in at least 4-5 genes are required and it is the total number of mutations rather than their sequence that is important for malignant transformation to occur. Transformation from the initial events to an invasive carcinoma takes about 8-12 years. As colonic mucosa is a highly dynamic tissue and the mucosal surface epithelium cells are constantly replaced with cells derived from crypt stem cells; it is reasonable to assume that only the long-lived cells (stem cells) may serve as reservoirs for accumulation of such precancerous mutations. In a normal colon, these cells are typically present at the bottom of the colonic crypts[3]. Cancer stem cells (CSCs), that possess remarkable similarity with normal stem cells, are thought to be the result of accumulated mutations, specifically in tumor suppressor genes and/or oncogenes[4]. Like normal stem cells, CSCs are also able to proliferate indefinitely and also possess the property of pluripotency indicating their capability to differentiate into more than one cell lineages. Recent evidence show that CSCs are present in many malignancies, including CRC[4-6]. Self-renewing properties of CSCs allow these cells to form tumors representing the original tumor in immuno-compromised mice. Within the epithelial malignancies, CSCs were first identified in breast cancer and characterized by specific cell surface markers[4,6]. Since then, they have been reported in a multitude other human malignancies. It is suggested that these self-renewing, pluripotent cancer stem/stem-like cells may play a pivotal role in initiation, development and progression of colorectal carcinoma.

Data generated from several investigations from our laboratory have revealed a progressive rise in CSCs in the colon with advancing age[7,8]. Stem cells, present in all vertebrates, are constantly replenishing dying cells or regenerating damaged or injured tissue. With aging, DNA repair system has been shown to be impaired that results in an increased DNA damage. DNA damage leading to reduction in some stem cells through apoptosis can result in genetic and epigenetic changes in stem cells that have survived the DNA repair mechanisms[9]. Both genetic and epigenetic alterations may affect stem cell function by altering transcriptome and lead to the processes of carcinogenesis (reviewed in[10]). Thus the age-related rise in CRC could partly be due a rise in CSCs.

CSCs can be identified by surface epitopes or their functional characteristics. Colon CSCs are characterized by the expression of several markers that represent the surface epitopes which among others include CD44, CD166, CD133 and EpCAM[11]. In addition, colonosphere formation is considered to be another functional assay for identification of CSCs.

Another characteristic of CSCs is the acquisition of epithelial to mesenchymal transition (EMT), which provides the cells ability to migrate, invade and metastasize. EMT can be determined by E-cadherin and vimentin expression, which are downstream targets of Wnt/β-catenin and notch signaling[12]. Our earlier data suggested a pivotal role fort Wnt/β-catenin signaling for proliferation and maintenance of CSCs in the colon[13]. Over-expression and/or induction of epidermal growth factor receptor (EGFR) signaling and/or other members of receptor tyrosine kinase family, especially ErbB-2 has also been shown to occur in many cancers including the colon and is considered to be an indicator of poor prognosis. We have postulated that activation of EGFR in the gastrointestinal tract may lead to stem cell proliferation and maintenance as inhibition of EGFR by cetuximab reduced CSCs in the colon[14].

In view of the recent evidence indicating the appearance of CSCs is one of the initial events in carcinogenesis, we have investigated and confirmed that age-related increases in adenomatous polyps are associated with increases in mucosal CSCs[7]. We demonstrated that with advancing age there is a progressive rise in CSCs in the colon not only in adenomas, but also in normal appearing mucosa. This observation indicates that aging increases the risk of CRC[7]. The number of colonic mucosal cells showing $CD44^+$, $CD166^+$ or EpCAM was markedly higher in the isolated mucosal cells in subjects over 55 years of age with polyps than the younger ones.

We also reported an age-related rise in expression and activation of all members of EGFRs with the exception of EGFR-4, which was not studied[15-19]. In addition, our data also revealed that CD166 and EGFR were co-localized in normal appearing mucosa of patients with adenomas. Interestingly, the co-expression of CD166 and EGFR was found to be markedly higher in individuals over 60 years

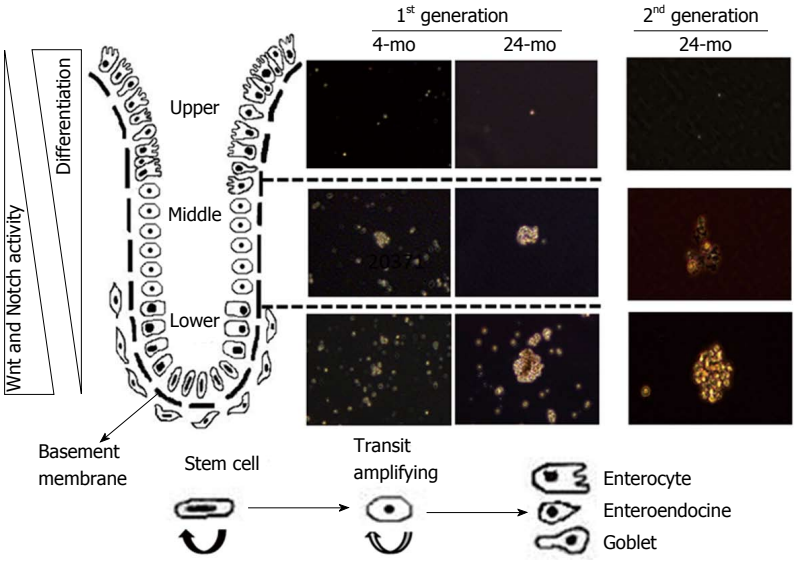

Figure 1 Changes in colonosphere forming potential of mucosal cells isolated from different regions of colonic crypt: Young (4-5 mo) and aged (22-24 mo) Fischer 344 rats were euthanatized by CO_2 asphyxiation following an overnight fast. The colon was removed, rinsed with cold PBS, everted, filled with a 5-10 mL protease solution [1 mg/mL collagenase 1 and 20 μg/mL hyaluronidase 1 in 0.05% Trypsin-EDTA (1X) with 2% BSA] and ligated at both ends. The colon was placed in 0.05% Trypsin-EDTA (1X) and incubated for 30 min at room temperature. To obtain the cells from the upper part of the colonic crypt, the colonic bag was transferred into 50 mL DMEM/F12 and incubated for 60 min at room temperature. For cells from the middle region of the crypt, the colonic bag was transferred into fresh 50 mL DMEM/F-12 and incubated at room temperature for another 45 min. Finally, the colonic bag was incubated further for 45 min at room temperature to obtain the cells from the lower part of the crypt. The dispersed mucosal cells were collected by centrifugation at 500 g for 5 min, washed with DMEM/F12, immediately suspended and cultured in serum-free stem cell medium containing DMEM/F12 (1:1) supplemented with B27, 20 ng/mL epidermal growth factor, 10 ng/mL fibroblast growth factor, 50 μg/mL gentamicin and antibiotic-anti-mycotic. First generation colonospheres were observed after 7 and 14 d. The colonospheres were collected, trypsinized and re-suspended in stem cell medium for formation of second generation colonospheres. PBS: Phosphate buffer saline; BSA: Bovine serum albumin.

of age suggesting that with aging risk of developing CRC increases[7]. Expression of CSC markers was also found to be higher in *Helicobacter pylori* gastritis[20] and gastric cancers and also in normal appearing gastric mucosa from the aged[21]. The precise underlying mechanisms for the age-associated increase in gastrointestinal malignancies, specifically CRC remain to be elucidated. We have hypothesized that CSCs, which are thought to arise from mutations of normal stem cells residing at the bottom of the crypt, will proliferate and migrate with time to occupy the entire crypt. This will eventually lead to the age-related rise in colon cancer. We tested this hypothesis by isolating mucosal cells from three different regions along the colonic crypt (upper, middle and lower 1/3) of young (4-mo) and aged (24-mo) Fischer-344 rats and subjecting them to colonosphere formation and mutational analysis. Our results showed that the number of spheroids formed by the mucosal cells isolated from the middle and lower regions of the crypt from aged animals were higher than their younger counterparts. No such difference was observed in cells isolated from the upper region of the colonic crypt between the two age-groups. In addition, we also found cells from the lower and middle regions of colonic crypt of older animals to form spheroids for another generation. Although mucosal cells, isolated from bottom of the crypt of young rats did form a few colonospheres inconsistently, they were also smaller in size. In contrast, mucosal cells isolated from the mid and upper parts of colonic crypt of young rats did not form spheroids (Figure 1). The increased colonosphere formation by mucosal cells from older animals was accompanied by a parallel rise in colonic CSC marker CD44 and also β-catenin, which is known to be dysregulated in colon cancer. On the other hand, the levels of the differentiation marker CK-20 in the middle and upper part of the crypts of older animals were markedly higher than the levels noted in the lower region. Likewise, colonic mucosal cells from the lower region of aged rats exhibited an increased frequency of mutations of the colonic crypt of than their younger counterparts.

In conclusion, our data demonstrate a gradual increase in CSCs in the colonic crypt with advancing age, which could partly contribute to the age-related rise in CRC. Although the underlying reasons for the rise in CSCs in the colon with advancing age remain to be fully explored, one possibility could be that aging renders the gastrointestinal mucosa more susceptible to ever-increasing environmental or other toxicants.

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EDITORIAL

Alcoholic hepatitis: The pivotal role of Kupffer cells

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Abstract

Kupffer cells play a central role in the pathogenesis of alcoholic hepatitis (AH). It is believed that alcohol increases the gut permeability that results in raised levels of serum endotoxins containing lipopolysaccharides (LPS). LPS binds to LPS-binding proteins and presents it to a membrane glycoprotein called CD14, which then activates Kupffer cells *via* a receptor called toll-like receptor 4. This endotoxin mediated activation of Kupffer cells plays an important role in the inflammatory process resulting in alcoholic hepatitis. There is no effective treatment for AH, although notable progress has been made over the last decade in understanding the underlying mechanism of alcoholic hepatitis. We specifically review the current research on the role of Kupffer cells in the pathogenesis of AH and the treatment strategies. We suggest that the imbalance between the pro-inflammatory and the anti-inflammatory process as well as the increased production of reactive oxygen species eventually lead to hepatocyte injury, the final event of alcoholic hepatitis.

Key words: Alcoholic liver disease; Alcoholic hepatitis; Macrophages; Kupffer cells

Core tip: In this editorial we provide critical comments on the pivotal role of Kupffer cells on the development of alcoholic hepatitis with a focus on the pro-inflammatory as well as the anti-inflammatory pathways. We propose that the anti-inflammatory pathway should be further explored as a potential alternative for novel treatment strategies. This editorial is significant as it provides a platform for the future basic and clinical

research in elucidating the pathogenesis and developing the management strategies of this common clinical pathology - alcoholic hepatitis.

Suraweera DB, Weeratunga AN, Hu RW, Pandol SJ, Hu R. Alcoholic hepatitis: The pivotal role of Kupffer cells. *World J Gastrointest Pathophysiol* 2015; 6(4): 90-98 Available from: URL: http://www.wjgnet.com/2150-5330/full/v6/i4/90.htm DOI: http://dx.doi.org/10.4291/wjgp.v6.i4.90

INTRODUCTION

Alcoholic hepatitis (AH) is defined as an acute hepatic inflammatory response to excess alcohol ingestion. It is estimated that 56809 hospital admissions in 2007 in the United States had a primary diagnosis of AH, 0.71% of all admissions[1]. In addition, hospitalization for AH is a leading cause of healthcare utilization[1]. In spite of such high costs and mortality, there has been little progress in the treatment strategies over the past 20 years. Histologically, alcoholic hepatitis is characterized by hepatocellular necrosis and immune cell infiltration around damaged hepatocytes[2]. This inflammatory and immune response leads to further hepatic injury and acute liver failure. Thus understanding this inflammatory cascade is vital to understanding alcoholic hepatitis and developing a treatment strategy. Currently there are only two pharmacologic treatments of AH: Corticosteroids and pentoxifylline. However these treatments are limited in their effectiveness and severe cases of AH still carry a short term mortality of 30%-50%[3]. Hepatic macrophages, called Kupffer cells, have been found to play a central role in hepatic inflammation[4]. Therefore, we will focus on providing a concise review of the role of Kupffer cells in AH, current treatments to disrupt this inflammatory pathway and potential basic and clinical research directions.

OVERVIEW OF THE PHYSIOLOGIC FUNCTION OF KUPFFER CELLS

Kupffer cells are macrophages found in the liver. They were first identified by Kupffer[5] in 1876. Monocytes in the blood stream migrate into the liver and differentiate into Kupffer cells[6]. Kupffer cells makeup about 15% of all cells in the liver and comprise 50% of the total population of macrophages in the body[7]. They function to clear foreign matter from the portal circulation and in animal models have been shown to clear about 80%-90% of all particulate injected[8]. The particulate include immune complexes, bacterial components, endotoxins and collagen fragments. Kupffer cells can kill ingested organisms using oxygen dependent and independent mechanisms[9]. Studies in Kupffer cell depleted mice have shown that Kupffer cells play a critical role in neutrophil recruitment and granulomatous formation in the liver[10]. Kupffer cells are activated by endotoxins (Figure 1). Endotoxins are composed of the lipopolysaccharides (LPS) component of Gram-negative bacterial cell walls. LPS-binding proteins (LBPs), produced by hepatocytes, bind and present LPS to CD14, a membrane glycoprotein[9]. CD14 in turn activates Kupffer cells *via* a membrane complex that includes a pathogen recognition receptor called toll-like receptor 4 (TLR-4). Activated Kupffer cells release interleukin (IL)-1B, tumor necrosis factor (TNF)-α, IL-6, IL-8, macrophage chemotactic protein-1 and regulated normal T cell expressed and secreted. These cytokines, mainly TNF-α, then bind to hepatocyte receptors leading to tissue damage *via* oxidative stress and apoptosis[11].

ACTIVATION OF IMMUNE RESPONSE INALCOHOLIC HEPATITIS

Gut bacterial translocation likely plays a key role in AH. In a healthy individual, only a small quotient of gut bacterial endotoxin gets translocated into the portal blood. Alcohol ingestion has been shown to increase this endotoxin translocation[12]. Alteration of gut microflora and increased gut permeability are the driving forces behind this process. Experimentally induced bacterial overgrowth in rats has been shown to lead to increased bacterial translocation and subsequent liver injury[13]. Furthermore, evidence suggests that alcohol can alter gut microflora[14]. Jejunal aspirates of chronic alcohol abuse patients have shown increased aerobic and anaerobic bacteria[15,16]. The pathophysiology of bacterial overgrowth in chronic alcoholic patients is not clearly identified. Possible etiologies include impaired bile flow, reduced gastrointestinal motility and increased gastric pH[14,17-19]. In addition to bacterial overgrowth, alcohol can lead to intestinal dysbiosis. Animal studies have shown an increased predominance of Gram-negative bacteria in alcohol fed subjects[20,21]. Mice with antibiotic induced eradication of gut flora had decreased alcohol induced liver injury as compared to mice with intact gut flora when exposed to ethanol[22]. Similar results were found in mice that were fed with lactobacillus[23]. Intestinal decontamination with rifaximin has also shown increased liver hemodynamics and decreased incidence of hepatic encephalopathy in patients with alcoholic liver disease (ALD)[24,25]. The second component of alcohol induced endotoxemia is increased gut permeability. Alcohol is metabolized into acetaldehyde, which has been shown to open tight junctions and increase gut epithelium permeability[26,27]. Several studies have suggested the association between endotoxins and alcoholic liver injury. It was found that endotoxin levels in mice directly correlated with the severity of alcoholic liver injury[28]. Rats that had LPS administered in addition to alcohol were also shown to have worse liver injury than those exposed to ethanol alone[29]. In humans, endotoxin levels have been shown to be measurably higher in acute and chronic alcohol use[30].

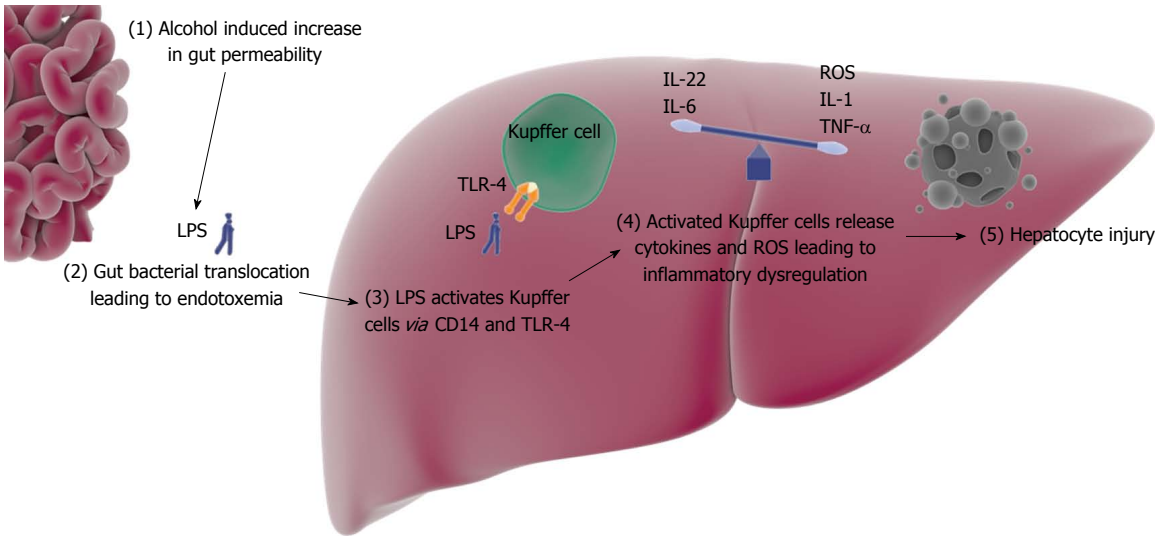

Figure 1 Central mediating role of Kupffer cells in alcoholic hepatitis. The dysregulation between the pro-inflammatory and the anti-inflammatory cytokines eventually leads to hepatocyte injury. Image components obtained from somersault 1824 online image library (http://www.somersault1824.com/). LPS: Lipopolysaccharides; TLR-4: Toll-like receptor 4; IL: Interleukin; TNF: Tumor necrosis factor; ROS: Reactive oxygen species.

IMPORTANCE OF KUPFFER CELLS IN ALCOHOLIC HEPATITIS

Several lines of evidence suggest that Kupffer cells play an important role as inflammatory mediators in the setting of alcoholic hepatitis. TLR-4 defective rats exposed to ethanol were shown to have markedly less steatosis, inflammation, and necrosis as compared to wild-type rats[31]. Furthermore ethanol increased TNF-α in wild-type rats but failed to do so in the TLR-4 mutant rats[31]. In LBP and CD14 knockout mice, alcohol induced liver injury was also significantly reduced[31-33]. Mice in whom Kupffer cells were chemically destroyed had no alcohol induced liver injury[34]. Activated human Kupffer cells express CD163, a hemoglobin-haptoglobin scavenger surface receptor[35]. Although the function of CD163 is unknown, it has been used as a marker for macrophage activation. Studies have shown that CD163 is in fact not only elevated in ALD, but that the plasma concentration of CD163 also predicts mortality in acute liver failure[36]. In addition CD163 has been shown to be a predictor of clinical decompensation in the setting of liver cirrhosis, an independent prognostic indicator for variceal bleeds and a marker of portal hypertension[37-39]. It is important to note that a recent study comparing levels of CD163 in AH, chronic cirrhosis and healthy patients found that CD163 concentrations were 30% higher in AH patients than in chronic cirrhotic patients and 10 times higher as compared to healthy individuals[40]. Therefore, CD163 could serve as a diagnostic marker of alcoholic hepatitis as well as a potential prognosticator for patients with alcoholic hepatitis.

Kupffer cell-mediated products have been extensively studied to further characterize their association in AH. TNF-α has been identified as a key mediator in AH. Serum TNF-α have been found to correlate with endotoxemia and development of inflammation and fibrosis in patients with AH. It can even be used as a biomarker for fibrosis[41,42]. Studies have confirmed that monocytes from patients with alcoholic hepatitis had greater levels of TNF-α than healthy subjects[43]. Furthermore, analysis of liver biopsies in patients with AH have shown increased staining for TNF-α, IL-1 and IL-6[44]. Kupffer cells can also contribute to liver injury *via* oxidant stress. Kupffer cells in animals fed with alcohol produce free radicals. This is further supported by studies showing nicotinamide adenine dinucleotide phosphate oxidase knocked out mice demonstrated to have decreased liver necrosis and inflammation in addition to decreased nuclear factor-kappa B and TNF-α[45].

In addition to the resident Kupffer cell-mediated hepatic injury, recruited macrophages have also been shown to play a part in liver injury[46]. Murine models have shown that there is an increased accumulation of infiltrating monocytes in the setting of liver injury[47]. Recruitment of these monocytes is highly dependent on the chemokines CCL1 and CCL2. Of note, one of the major sources of CCL2 is hepatic stellate cells, which in turn are activated by the TLR-4 ligands. Mice lacking CCL2 have been shown to incur less liver injury[48]. Furthermore mice lacking CCR8, a receptor for CCL1, were also shown to be more protected from liver injury[49]. Infiltrating monocytes have been divided into two groups depending on surface protein expression, $Ly6C^{hi}$ and $Ly6C^{low}$. $Ly6C^{hi}$ monocytes exhibit a pro-inflammatory phenotype while $Ly6C^{low}$ monocytes exhibit an anti-inflammatory phenotype. Mice fed with ethanol had a shift towards more $Ly6C^{hi}$ monocytes, resulting in significantly increased liver injury[50]. There is still much to be learned about the role and function of infiltrating monocytes in liver injury.

Kupffer cells have been shown to play central roles in other causes of liver injury such as nonalcoholic

Table 1 Randomized controlled trials evaluating the treatment of alcoholic hepatitis

Study	Topic	Methods	Findings
Prednisolone or pentoxifylline			
Theodossi *et al*[99]	PRED *vs* placebo	Randomized control	No difference in mortality
Ramond *et al*[100]	PRED *vs* placebo	Double-blinded, randomized control	Improved mortality with PRED
Akriviadis *et al*[69]	PTX *vs* placebo	Double-blinded, randomized control	Improved mortality with PTX
Sidhu *et al*[101]	PTX *vs* placebo	Randomized control	Improved mortality with PTX
De *et al*[102]	PTX *vs* PRED	Double-blinded, randomized control	Reduced mortality with PTX
Park *et al*[103]	PTX *vs* PRED	Randomized control	Reduced mortality with PRED
Mathurin *et al*[104]	PRED *vs* PRED + PTX	Multicenter, double-blinded, randomized control	No difference in mortality
De *et al*[105]	PTX *vs* PTX + PRED	Double-blinded, randomized control	No difference in mortality
Thursz *et al*[70]	PTX *vs* PRED *vs* placebo	Multicenter, double-blinded, randomized control	No difference in mortality
N-acetylcysteine			
Moreno *et al*[106]	NAC *vs* placebo	Multicenter, single-blinded, randomized control	No difference in mortality
Cytokine inhibitors			
Naveau *et al*[72]	Infliximab *vs* placebo	Double-blinded, randomized control	Increased mortality with infliximab
Boetticher *et al*[71]	Etancercept *vs* placebo	Multicenter, single-blinded, randomized control	Increased mortality with etancercept

PTX: Pentoxifylline; PRED: Prednisolone; NAC: N-acetylcysteine.

steatohepatitis (NASH) and viral hepatitis that are often also present in AH patients. Using a murine model of NASH, several studies have shown that sequential depletion of Kupffer cells reduced the incidence of steatosis[51-53]. Furthermore, targeted knockdown of TNF-α also decreased the incidence of NASH development[51,54]. Current understanding of the role of Kupffer cells in viral hepatitis is limited. Identification of a specific pathogenesis has been difficult due to similar characteristics of recruited macrophages and resident Kupffer cells. A recent study suggests that Kupffer cell interaction with hepatitis B surface antigen leads to pro-inflammatory cytokine production, which may contribute to liver pathology[55]. Studies have shown increased numbers of Kupffer cells during hepatitis C viral (HCV) infection[56]. Incubation of HCV E2 envelop protein with human liver cells resulted in Kupffer cell binding in a CD81-dependent manner[57]. In addition HCV core and NS3 stimulate human $CD14^+$ Kupffer cells and monocyte derived macrophages to produce IL-1β, IL-6 and TNF-α[58,59]. It is likely that Kupffer cell activation contributes to the progression of liver disease in viral hepatitis. Increased numbers of Kupffer cells have been found in regions of liver fibrosis in the setting of chronic viral hepatitis[60]. Viral hepatitis has also been shown to induce Kupffer cells to release cytotoxic molecules that kill not only infected hepatocytes but also non-infected cells[61,62]. It is likely that Kupffer cells are involved in the pathogenesis of many types of liver pathologies and it may be the case that their activation is multifactorial in patients with AH as well as other hepatic comorbidities.

CURRENT TREATMENT OF ALCOHOLIC HEPATITIS

AH is an acute process and most patients will recover with nutritional support and abstinence from alcohol. However severe AH carries a high mortality rate: 35% at 28 d without effective treatment[63]. These high mortality rates are predominantly due to a lack of effective treatment for severe AH. Multiple clinical trials for treatment of alcoholic hepatitis have been published (Table 1). The American Association for the Study of Liver Diseases (AASLD) guidelines for management of AH currently stratifies the management depending on severity. Low risk patients are managed conservatively with nutrition, supportive care and close monitoring. High-risk individuals, defined as those with a Maddrey's discriminant function greater than or equal to 32 or a model for end-stage liver disease score greater than or equal to 18, may benefit from pharmacological intervention with either prednisolone or pentoxifylline. Corticosteroids have been extensively studied with mixed results[63-67]. This is likely due to the fact that study design, severity of AH and exclusions criteria vary greatly between studies. One meta-analysis showed survival rates of 80% at 28 d with corticosteroids *vs* 66% in the control group in patients with severe AH[63]. Corticosteroids presumably improved outcomes by decreasing pro-inflammatory cytokines. Pentoxifylline is a nonselective phosphodiesterase inhibitor that increases intracellular concentration of adenosine 3', 5'-cyclic monophosphate, which in turn inhibits the expression of pro-inflammatory cytokines[68]. AASLD recommends pentoxifylline as an alternative to corticosteroids when the use of steroids is contraindicated or in the setting of early renal failure. According to one randomized, double-blinded, placebo controlled trial, patients treated with pentoxifylline had a survival benefit (24.5% mortality *vs* 46.1% in the placebo group)[69]. Although multiple clinical trials have shown some benefit of treatment with steroids or pentoxifylline, a recent well designed, multicenter, double-blinded, randomized trial found no statistically significant mortality benefit in treatment with either pentoxifylline or prednisolone[70]. The study involved 1053 patients who were randomized to four arms: A group that received a pentoxifylline-matched placebo and a prednisolone-matched placebo, a group that received prednisolone and a pentoxifylline-matched

placebo, a group that received pentoxifylline and a prednisolone-matched placebo, or a group that received both prednisolone and pentoxifylline. The prednisolone group was the only group associated with an initial reduction in 28-d mortality. However at 90 d and at 1 year there were no significant differences between the groups. There is no doubt that this well designed study certainly questions the currently established treatments of AH.

While cytokine inhibitors have great potential in theory, trials with both infliximab and etanercept have resulted in increased mortality, primarily due to infection[71,72]. Liver transplantation is another treatment option in ALD. Most transplant centers require at least 6-months of abstinence[73,74]. This allows for disease regression in patients with recent alcohol use, time for proper counseling and demonstrates patients' ability to abstain from alcohol. One meta-analysis comparing alcohol use in post-transplant patients showed no difference in the proportion of patients that used alcohol when comparing ALD to non-ALD patients, although ALD patients were more likely to drink excessively[75]. Risk of alcohol recurrence in ALD transplant patients continues to be an area of debate. In summary, treatment options for AH are limited with even the standard of care now being questioned, emphasizing the urgent need for effective and novel treatment strategies.

FUTURE AREAS OF RESEARCH

Identification of new therapeutic targets has been hampered by a lack of appropriate animal models. Current animal models do not develop severe liver injury as humans do. One possible area of future investigations would be the modulation of the LPS pathway. A recent study evaluating the effects of milk osteopontin on gut permeability found that milk osteopontin preserved gut architecture and prevented inflammation in ethanol fed mice[76]. Milk osteopontin has also been shown to directly bind to LPS and prevent Kupffer cell activation thereby disrupting the subsequent pro-inflammatory cascade[77]. Another study used probiotics to alter gut flora and TLR4 antagonists, which have been proposed for treatment of ALD[78].

Genetic factors leading to the predisposition for liver disease is another promising area of exploration in recent years. A number of studies have shown an association between variations in the *PNPLA3* gene and liver fat content as well as plasma aspartate aminotransferase[79-82]. Furthermore two groups have independently found associations between the PNPLA3 single-nucleotide polymorphism rs738409 and ALD populations in Mexico and Germany[83,84]. During the last decade, a prominent area of research had been the inhibition of pro-inflammatory cytokines. However blocking TNF-α had led to unacceptable complications. More targeted inhibition using dexamethasone conjugates targeting the CD163 receptor on macrophages have shown some success in rats[85,86]. Yet another unique way of managing inflammation in AH patients is apheresis. A recent case series and literature review of 35 cases concluded that leukocytapheresisand granulocytapheresiswere effective in controlling leukocytosis as wells as inflammatory cytokines[87].

In contrast to pro-inflammatory cytokines, Kupffer cells also produce anti-inflammatory or hepato-protective cytokines, such as IL-6 and IL-22[88] (Figure 1). Activated Kupffer cells release IL-6, which then stimulates signal transducer and activator of transcription 3 (STAT3) leading to increased expression of genes that are anti-apoptotic, anti-oxidative, and promote mitochondrial DNA repair[89,90]. Studies have shown that IL-6 deficient mice are in fact more susceptible to hepatic steatosis, cellular apoptosis and mitochondrial DNA damage when exposed to ethanol[89-91]. Furthermore STAT3 knockout mice have been shown to have greater degree of hepatic steatosis as compared to wild-type mice[92]. Ethanol induced liver injury was alleviated by treatment with IL-6[93]. IL-22 is another hepato-protective cytokine that has been found to ameliorate hepatocellular damage in fatty liver as well as acute and chronic alcoholic liver injury[94-97]. It is believed that both IL-6 and IL-22 share the same pathway, STAT3 mediated hepatoprotection[96].

Another potentially important observation relevant to alcoholic hepatitis is a recently reported finding that the administration of lactate reduced inflammation and organ injury in mice with an immune mediated hepatitis[98]. Lactate interacted with the specific receptor G protein-coupled receptor 81 (GPR 81) to reduce inflammation and injury. Further, lactate and GPR 81 prevented LPS-induced macrophage activation (Kuppfer cells) suggesting that the beneficial effects were mediated by the effects of lactate on activated macrophages. These results suggest that hepatic injury due to macrophage activation may be treated by ligands including lactate that interact with GPR 81.

CONCLUSION

AH is a major cause of morbidity and mortality worldwide. The underlying mechanisms are poorly understood, which has resulted in a lack of specific treatments. The absence of animal models further hampered the progress in elucidating the molecular mechanisms which may provide scientific evidence for designing more targeted treatment strategies. Given the inconsistent results of currently available treatment strategies, which mainly target the pro-inflammatory process, we speculate that it is also important to recognize the potential effort of targeting the anti-inflammatory pathway or targeting both the anti and the pro-inflammatory pathways simultaneously. With the recognition of the anti-inflammatory process mediated by Kupffer cells, it may be the prime time for a well-designed clinical trial to target the unique anti-inflammatory pathway. This may lead to the development of novel effective treatment strategies for

this common clinical entity.

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EDITORIAL

Starring role of toll-like receptor-4 activation in the gut-liver axis

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Abstract

Since the introduction of the term "gut-liver axis", many studies have focused on the functional links of intestinal microbiota, barrier function and immune responses to liver physiology. Intestinal and extra-intestinal diseases alter microbiota composition and lead to dysbiosis, which aggravates impaired intestinal barrier function *via* increased lipopolysaccharide translocation. The subsequent increased passage of gut-derived product from the intestinal lumen to the organ wall and bloodstream affects gut motility and liver biology. The activation of the toll-like receptor 4 (TLR-4) likely plays a key role in both cases. This review analyzed the most recent literature on the gut-liver axis, with a particular focus on the role of TLR-4 activation. Findings that linked liver disease with dysbiosis are evaluated, and links between dysbiosis and alterations of intestinal permeability and motility are discussed. We also examine the mechanisms of translocated gut bacteria and/or the bacterial product activation of liver inflammation and fibrogenesis *via* activity on different hepatic cell types.

Key words: Gut microbiota; Dysbiosis; Toll-like receptor 4; Gut motility; Lipopolysaccharide tolerance; Non-alcoholic fatty liver disease; Chronic hepatitis; Intestinal barrier function; Liver fibrosis

Core tip: Liver disease is associated with significant changes in intestinal microbiota, but whether liver disease modifies the complement of gut bacteria or dysbiosis causes liver disease is not clearly understood. This review outlines current knowledge on the gut-liver axis, with a particular focus on the role of toll-like receptor 4 activation in functional gastrointestinal disorders, liver inflammation and fibrosis.

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INTRODUCTION

The term gut-liver axis was introduced approximately 40 years ago, when Volta *et al*[1] described the production of IgA antibodies directed against intestinal microorganisms and food antigens in liver cirrhosis. The functional link between the gut and liver has been extensively investigated since this first report[1]. Intestinal microbiota, barrier function and immune responses that link the gut and liver are intriguing and promising research topics.

Growing evidence demonstrates that gut microbiota play an important role in the gut-liver axis[2]. Disturbances in gut microbiota composition may contribute to many diseases and affect local and remote organ systems[3]. Several conditions are associated with specific microbial patterns and/or leaky gut. These disorders range from intestinal diseases, such as irritable bowel syndrome and inflammatory bowel diseases, to numerous extra-intestinal diseases[3], including diseases that affect the liver[4]. The intestinal mucosa exhibits impaired barrier function in the presence of abnormal microbiota, such as increased intestinal permeability and endotoxin translocation, with the subsequent increased passage of waste materials from the intestinal lumen to the organ wall and bloodstream[2,5]. The gut epithelium is a natural barrier that allows the selective entry of substances present in the lumen and avoids the entry of harmful elements, including bacteria and their bioproducts[6].

Toll-like receptors (TLRs) are a family of highly conserved receptors that recognize pathogen-associated molecular patterns and allow the host to recognize bacteria, mycobacteria, yeast membrane/wall components and several gut-derived products. TLR-4 is one of the most intriguing of these receptors because it plays a key role in innate immunity by triggering inflammatory responses. TLR-4 initiates innate immune responses *via* nuclear factor kappa B (NF-κB) when it is activated by its primary ligand, Gram-negative bacterial lipopolysaccharides (LPS), which results in the transcription of several genes that encode inflammatory cytokines, chemokines and antimicrobial agents[7,8].

This review analyzed the most recent literature on the gut-liver axis, with a particular focus on the role of TLR-4 activation. First, we evaluated the evidence that links liver disease with the condition of dysbiosis. Second, we discuss the links between dysbiosis and alterations in intestinal permeability and motility. Finally, we examine the mechanisms of translocated gut bacteria and/or the bacterial product activation of liver inflammation and fibrogenesis *via* activity on different hepatic cell types.

DYSBIOSIS DURING CHRONIC LIVER DISEASE AND CIRRHOSIS

Liver disease is associated with significant qualitative and quantitative changes in intestinal microbiota, which is defined as "dysbiosis". Dysbiosis is directly involved in the pathogenesis of several different forms of hepatic injury and many complications of advanced cirrhosis. Whether liver disease modifies the complement of gut bacteria or dysbiosis causes liver disease is not clearly understood. Existing evidence supports the need to contextualize the argument within the etiology of liver disease. Conversely, advanced liver disease is associated with dysbiosis that is independent from the original cause of hepatic damage. The hypothesis of a vicious circle in which microbiota alterations are supported by cirrhosis, which contributes to many cirrhosis complications seems appropriate and well structured.

Most of the research on dysbiosis during chronic liver disease investigated non-alcoholic fatty liver disease (NAFLD). Qualitative and quantitative dysbiotic changes are clearly documented during NAFLD in patients with simple fatty liver and non-alcoholic steatohepatitis (NASH). NAFLD patients exhibit a high prevalence of small intestinal bacterial overgrowth[9,10], and microbial samples from NAFLD and NASH patients exhibit a significantly lower proportion of members of the *Ruminococcaceae* family than healthy subjects[11]. Some conflicting results emerged in studies that compared the microbiota between NAFLD and NASH patients, and further studies are anticipated on this subject.

The feeding of a high-fat/high-polysaccharide or a calorie-restricted diet to wild-type mice significantly alters microbial taxonomic composition in experimental models[12,13], and gut microbiota exacerbate NAFLD development *via* several different mechanisms. First, gut microbes participate in calorie extraction from food and regulate obesity and its complications, including NAFLD. Human enzymes cannot degrade most complex carbohydrates and plant polysaccharides, which are fermented in the colon by intestinal microbes. The resultant short-chain fatty acids account for approximately 10% of daily energy intake[14] and stimulate *de novo* lipogenesis[15]. The intestinal microflora is also responsible for the increased endogenous ethanol production that is observed during NAFLD. An age-related increase in breath ethanol content was reported in *ob/ob* mice, and neomycin treatment abolished this effect[16]. Increased systemic ethanol levels were also confirmed in NASH patients[17], which may contribute to hepatocyte trygliceride accumulation and reactive oxygen species production. Bacterial conversion of dietary choline into methylamines experimentally produced similar effects of choline-deficient diets and caused NASH[18]. More recently, gut microbiota, which are responsible for

the conversion of cholic and chenodeoxycholic acid into secondary bile acids, were suggested to control lipid and glucose metabolism through the regulation of bile acid pools. Bile acids also function as signaling molecules and bind to cellular receptors. For example, bile acid synthesis controls the activation of nuclear receptor farnesoid X receptor and the Takeda G-protein-coupled receptor 5[19,20], which are strongly implicated in the modulation of glucose metabolism[21,22]. Hepatotoxic bacterial products that pass across a dysregulated intestinal barrier trigger liver damage, as discussed below, and provoke systemic inflammation and insulin resistance[23], which is a primary event in NAFLD pathogenesis. Circulating levels of LPS, which is a component of the outer membrane of Gram-negative bacteria, are elevated in rodent NAFLD[24,25] and NAFLD patients[26,27].

Research on the role of the microbiome in alcoholic liver disease is not as advanced as NAFLD, but dysbiosis is clearly associated with alcohol-induced liver damage. Significant microbial alterations were observed in the Tsukamoto-French model of alcoholic liver disease in mice[28], and *Lactobacilli* administration reduced the features of alcoholic liver disease in several animal models[29,30]. Significant changes in the composition of the microbiome are also observed in alcoholic patients, which is consistent with these experimental results[31,32]. There are several mechanisms by which alcohol may contribute to dysbiosis. Commensal flora produce and metabolize ethanol, and alcohol intake may influence the complement of bacteria. Alcohol also produces intestinal dysmotility, alters gastric acid secretion and impairs the intestinal innate immune response[33].

Dysbiosis is closely associated with advanced liver disease, *e.g.*, liver cirrhosis. An apparent increase in potentially pathogenic bacteria occurs during cirrhosis independently of the etiology of liver disease, with a greater abundance of Gram-negative taxa (*Enterobacteriaceae*, *Bacteroidaceae*)[34,35]. Similar to alcohol abuse, impaired intestinal motility and innate immunity may represent a basis for the dysbiosis that is observed during cirrhosis[36]. Cirrhotic patients are frequently exposed to hospitalization, antibiotics and dietary modifications, which are potential factors associated with alterations in the intestinal microbiome.

DYSBIOSIS: BARRIER DAMAGE, BACTERIAL TRANSLOCATION AND INTESTINAL DYSMOTILITY

Experimental models suggest that dysbiosis itself contributes to intestinal inflammation and mucosal leakage, which favors the translocation of several inflammatory bacterial products[37,38]. Intestinal decontamination with non-absorbable antibiotics also significantly reduces intestinal inflammation and permeability[38].

Intestinal barrier damage allows bacterial translocation, which is defined as the migration of viable microorganisms and microbial products (*e.g.*, LPS, lipoteichoic acid, bacterial DNA) across the intestinal barrier, from the intestinal lumen to mesenteric lymph nodes and other extra-intestinal organs and sites[39]. The translocation of viable bacteria may induce "spontaneous" bacterial infections in some cases, such as the spontaneous bacterial peritonitis that is observed during cirrhosis. The translocation of bacterial products that enter the systemic circulation *via* the portal vein and activate inflammatory pathways of hepatic cells contributes to the progression of liver damage in other cases, as discussed below. Viable microbes and bacterial fragments entering the systemic circulation *via* the portal vein or following the enteric lymphatic drainage trigger a proinflammatory state by provoking the release of cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and IL-1β, which contributes to the hyperdynamic circulation and portal hypertension that are typical of advanced liver cirrhosis[40]. Recent evidence suggests that intestinal barrier damage is due to a microbial imbalance that influences gut motility[41]. The observations of intestinal dysmotility in germ-free animals further suggest that microbiota play a crucial role in the modulation of intestinal motility[42]. TLRs may explain how microbiota act on gut motility and the gut-liver axis because TLR activation during conditions of impaired intestinal barrier mediates intestinal and liver disorders. Intestinal disorders that are associated with impaired motility may be caused by intestinal dysbiosis[41], which further increases intestinal permeability and the translocation of bacterial substances, especially LPS, that may reach the liver[5] (Figure 1).

The importance of aberrant intestinal microbiota in the pathogenic mechanisms of several gastro-intestinal diseases was raised previously, in addition to its health-inducing effects[2]. Commensal microbiota provides beneficial effects, including neuroimmune and pain modulation, and a possible effect on intestinal motility modulation. Polymicrobial sepsis induces a complex inflammatory response within the intestinal muscularis with the recruitment of leukocytes and the production of mediators that inhibit intestinal muscle function[43]. Therefore, the intestine is a source of bacteremia and an important target of bacterial products that affect intestinal motility[43]. Barbara *et al*[42] suggested in a recent paper that one of the possible mechanisms of microbiota influence on gut motor function occurs through the release of bacterial substances and the effects of mediators released by the gut immune response[42]. These inflammatory changes are partially determined by IL-1β mucosal expression, which is higher in patients suffering from post-infective irritable bowel syndrome (PI-IBS) than in patients without post-infectious symptoms[44]. Patients with IBS present increased IL-1β expression by peripheral blood mononuclear cells[45], and prolonged exposure to IL-1β alters neurotransmitter and electrically induced Ca^{2+} responses in the myenteric plexus[46]. The immune response also includes the release of histamine, tryptase and prostaglandins by mucosal-activated mast cells in PI-IBS[41] or activated macrophages during sepsis.

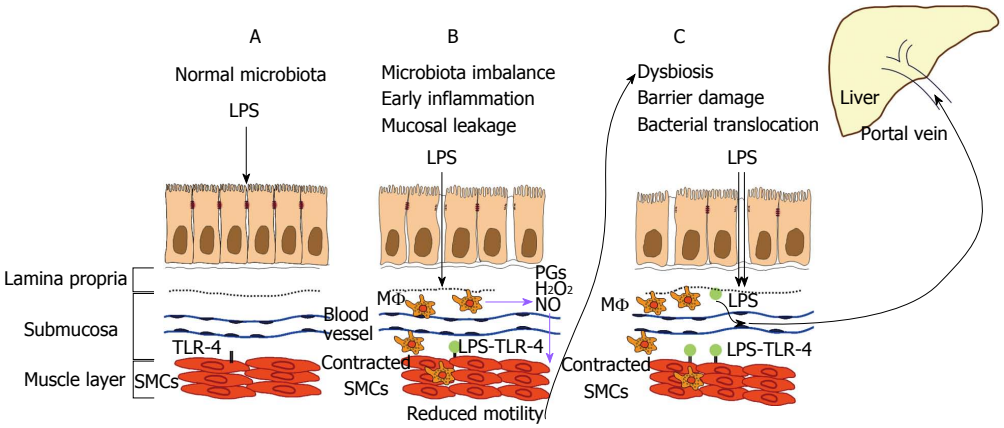

Figure 1 The results of intestinal disorders that are associated with impaired motility. A: Normal conditions with intestinal mucosa tolerance; B: In the presence of microbiota imbalance, intestinal mucosa is characterized by a leak and mild inflammatory infiltrate. The subsequent passage of modest quantities of lipopolysaccharides (LPS) induces activation of resident macrophages (MΦ) with the release of inflammatory mediators, such as prostaglandins (PGs) and nitric oxide (NO). LPS can also reach the muscle layer and bind to smooth muscle cell (SMC) toll-like receptor 4 (TLR-4). Both conditions cause morpho-functional changes of SMCs. The reduced intestinal motility further induces intestinal microbiota imbalance, which leads to dysbiosis; C: Dysbiosis induces barrier damage and relevant bacterial and LPS translocation. The large amount of translocated LPS reaches the blood vessels through the portal vein and reaches the liver.

Increased mucosal permeability was widely demonstrated during the course of sepsis and cases of severe mucosal inflammation[5], and impairment of contraction in these conditions seems to be related to the activation of normally quiescent intestinal muscularis macrophages by LPS or inflammatory mediators released by the mucosa[47-49]. Activated macrophages secrete several mediators, including prostaglandins, H_2O_2, cytokines and nitric oxide. Many of these mediators also alter the kinetic properties of smooth muscle cells (SMCs)[49-51]. Cyclooxygenase (COX)-1 and COX-2 are expressed in the neuromuscular compartment of the human colon, and these enzymes appear to modulate the cholinergic excitatory control of colonic motility at prejunctional and postjunctional sites, respectively[52]. IL-1β induces a decrease in tonic contraction in rat mesenteric lymphatic muscle cells in a COX-2 dependent manner *via* prostaglandin E_2[53].

Scirocco *et al*[54] demonstrated the constitutive expression of functionally active TLR-4 on primary human colonic SMCs in an *in vitro* model. Notably, exposure of SMCs to LPS caused contractile alterations[54]. This result suggests that the gastrointestinal dysmotility that occurs during acute infection is related to inflammation and a direct effect of circulating LPS on SMCs. TLR-4 activation following LPS binding leads to NF-κB activation, which participates in oxidative-dependent transcriptional changes in SMCs that modify the agonist-induced contraction[49,54]. LPS may also directly affect muscle cell contractility *via* alterations in electro-mechanical coupling[54], which could trigger a wide cascade of intracellular events that modify SMC integrity and function. Our group recently demonstrated that acute exposure of the human colonic mucosa to pathogenic LPS[49] impairs muscle cell contractility, and this effect was due to LPS translocation, which directly affects smooth muscle contractility, or the mucosal production of free radicals and inflammatory mediators that reach the muscle layer[49].

Notably, modulation of the intestinal microflora balance using probiotics likely plays an important role in the treatment and prevention of various gastrointestinal disorders[55]. The specific mechanisms underlying probiotic efficacy are not clearly elucidated, but most gastrointestinal diseases in which probiotics exhibit efficacy are associated with non-specific alterations of gastrointestinal motility, which suggests that the modulation of intestinal motility is another possible mechanism for the benefits of probiotic[55]. For example, *Lactobacillus paracasei* attenuated persistent muscle hypercontractility of jejunal strips in an animal model of PI-IBS[56], and *Bifidobacterium* and *Lactobacillus*, but not *Streptococcus*, alleviated visceral hypersensitivity and recovered intestinal barrier function and inflammation in a recent study in the PI-IBS mouse model, which correlated with an increase in tight junction proteins[57], such a claudin-1 and occludin. One of the mechanisms that underlies the altered permeability in IBS includes changes in the expression, localization and function of tight junctions[58]. Decreased levels of zonule occludin-1 (ZO-1) protein expression and disruption of claudin-1, occludin and ZO-1 expression were found in the apical region of the enterocytes during the course of IBS[59,60]. An increased risk of developing PI-IBS was also conferred by single nucleotide polymorphisms in the that gene encodes the tight junction protein E-cadherin[61].

Our group demonstrated that exposure of human colonic mucosa to *Lactobacillus rhamnosus* GG (LGG) may affect smooth muscle contraction, suggests that the modulation of muscle contractility represents a possible mechanism of action of these bacteria[62]. Notably, LGG acts through the direct activation of the Gram-positive sensing TLR-2, which is expressed on the surface of human colonic SMCs. We recently demonstrated that the surface expression of TLR-2 in resting cells was significantly decreased in cells exposed to LGG. This reduction in available receptors for monoclonal anti-TLR-2

binding further suggests the occurrence of an interaction of LGG with TLR-2 receptors. TLR-2 activation likely induces transitory myogenic changes with alterations in morpho-functional parameters in muscle tissue and isolated SMCs[63]. TLR-2 activates an intrinsic myogenic response that likely counteracts the damage that is induced by the pro-inflammatory burst from pathogen LPS on human gastrointestinal smooth muscle[63]. LGG likely protects human SMCs from LPS-induced damage *via* LGG binding to TLR-2, and TLR-2 activation leads to IL-10-mediated anti-inflammatory effects.

TLR-4-EXPRESSING CELLS AND SIGNALING IN THE LIVER

Inflammation during chronic liver damage correlates with fibrosis progression, but the molecular mechanism that links inflammation and fibrosis are not definitively understood. Several factors that participate in inflammation and liver fibrosis at the molecular and cellular levels were mentioned, regardless of the specific etiology involved. One of the pathways that has attracted the most attention in recent years as the putative link between liver inflammation and fibrosis is regulated by TLR-4 activation.

Several cell types express TLR-4 in the liver, including Kupffer cells, hepatic stellate cells (HSCs), biliary epithelial cells, hepatocytes and liver sinusoidal endothelial cell (LSECs)[64]. TLR-4 expression in healthy liver tissue is generally low because of the high degree of tolerance of this organ to the continuously incoming gut-derived TLR-4 ligands. The liver receives high concentrations of gut-derived endotoxin because of its location between the systemic and portal bloodstream and the connection with the intestine through the biliary tract. Kupffer cells and hepatocytes take up the incoming LPS, which removes it from the blood and places it into the bile[65-67]. Increased TLR-4 expression is induced in the injured liver, and inflammatory signaling cascades are triggered by this activation[68]. Two microRNAs are primarily involved in the regulation of "LPS tolerance". TLR-4 activation increases miR-155 levels, which leads to the degradation of Src homology 2 domain-containing inositol-5-phosphatase 1, a down-regulator of TLR-4 signaling, and stimulation of the TLR-4 signaling pathway[69]. However, TLR-4 activation increases miR-21 expression, which upregulates IL-10 *via* programmed cell death protein 4 inhibition[70]. TLR-4-induces IL-10, which inhibits miR-155 and downgrades TRL-4 signaling. Therefore, the balance between miR-21 and miR-155 likely plays a pivotal role in the regulation of "LPS tolerance". Other microRNAs are as fundamental in the control of the TLR-4-induced inflammatory response, particularly miR-146a and miR-9, which resolve the pro-inflammatory response by targeting key components in the TLR-4 signaling pathways, and miR-147, which promotes an anti-inflammatory response *via* repression of cytokine production[71].

Once normal immune tolerance is exceeded, LPS directly activates the TLR-4 signaling pathway on Kupffer cells, HSCs, hepatocytes and cholangiocytes (Figure 2). LPS cooperates with circulating LPS-binding protein and binds to TLR-4 on the plasma membrane of cells with two co-receptors [CD14 and myeloid differentiation protein (MD)2] to activated TLR-4 signaling pathways in a myeloid differentiation factor (MyD)88-dependent or independent manner[72]. The MyD88 dependent signaling pathway primarily uses the iκB kinase and mitogen-activated protein kinase signaling pathways, which determines the activation of NF-κB and activator protein-1, respectively, and regulates the expression of pro-inflammatory cytokines and other genes related to immune functions[72]. The MyD88-independent signaling pathway is mediated by the Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β, which activates interferon regulatory factor 3 and induces the expression of interferon (IFN)-β and genes that respond to IFN[72].

LPS, *via* activation of TLR-4 and the consequent inflammatory cascade in target cells, plays a key role in the pathogenesis and progression of fatty liver of alcoholic and non-alcoholic origin[24,73]. Szabo *et al*[73] recently suggested that alcohol and its metabolites regulate the intestinal barrier and allow increased LPS blood concentrations to reach the liver *via* the portal blood and promote TLR-4-induced inflammation and liver damage. The molecular mechanisms triggered by the LPS/TLR-4 binding are likely crucial in NAFLD. Animal models of genetically induced obesity demonstrate an increased susceptibility to liver damage from endotoxin, and exposure to low doses of LPS also determines steatohepatitis development[74]. Animal models of diet-induced steatohepatitis also exhibit increased levels of portal endotoxemia and TLR-4 hepatic hyperexpression[24]. Probiotic treatment prevents the histological features of NASH in genetically obese animal models[75], which supports the hypothesis of the pathogenetic role of intestinal-derived bacterial products.

TLR-4 likely plays a role in viral hepatitis C, but the relationship between hepatitis C virus (HCV) and TLR-4 is quite complex. HCV infection directly induces TLR-4 expression[76] and may determine the loss of tolerance to TLR-4 ligands by monocytes and macrophages[77]. The TLR-4 signal may also regulate HCV replication[78]. Variants of the *TLR-4* gene modulate the risk for liver fibrosis in Caucasian patients with chronic HCV infection[79,80]. TLR-4 was also involved in the cooperation between HCV and alcohol towards liver damage and hepatic oncogenesis in the liver progenitor cell transplantation model[81].

Inflammation (with secretion of TNF-α and IL-6) and anti-viral effects (with secretion of IFN-β) are determined by TLR-4 activation, depending on whether the MyD88-dependent or independent pathway is induced, respectively[82]. The function of TLR-4 in LPS-stimulated proinflammatory responses of Kupffer cells is well characterized[76,77], but new insights were proposed

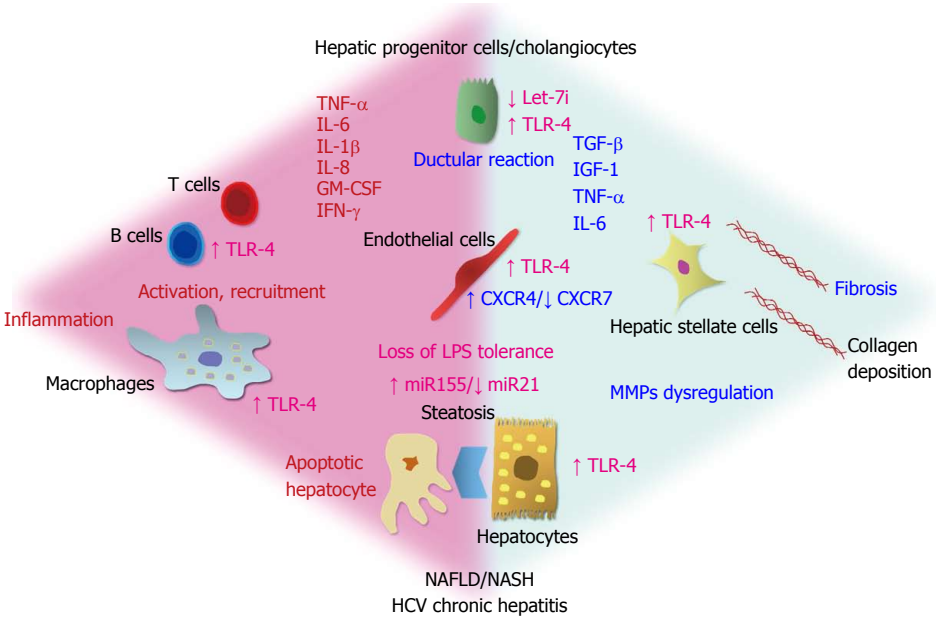

Figure 2 Hepatic cell types express toll-like receptor-4. In the presence of the loss of lipopolysaccharides (LPS) tolerance, such as during non-alcoholic fatty liver disease, non-alcoholic steatohepatitis or HCV chronic hepatitis, TLR-4 is activated by gut-derived LPS and overexpressed. An altered balance of known miRNAs (miR155, miR21, let-7i) and chemokine receptors (CXCR4, CXCR7) could promote this condition. Then, activation and recruitment of inflammatory cells, ductular reaction and activation of endothelial and stellate cells drive liver inflammation and fibrosis. On the right, the mediators mainly involved in the fibrosis are presented (TGF-β, IGF-1, TNF-α, IL-6), on the left, mediators related to the inflammation are shown (TNF-α, IL-6, IL-1α, IL-8, GM-CSF, IFN-γ). TLR-4: Toll-like receptor-4; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; HCV: Hepatitis C virus; MMPs: Matrix metalloproteinases; NAFLD/NASH: Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis.

recently. A TLR-4-driven metalloprotease expression has been postulated since matrix metalloproteinase (MMP)-10 was recently added to the list of genes that TLR-4 induces in liver macrophages[83]. MMP-10 was induced during hepatic injury and played a fundamental role in liver tissue repair[83]. Monocytes/macrophages represent the primary cellular targets of intestinal-derived endotoxin, and they are primary effectors of LPS-induced liver regeneration after partial hepatectomy and the experimental cholestatic liver disease, in which LPS promotes fibrogenesis[84,85].

TLR-4 expression by HSCs suggests a direct role of the receptor in hepatic fibrogenesis[72]. Transforming growth factor-β (TGF-β) signaling and liver fibrosis were enhanced by TLR-4 expression in HSCs[86], and the apoptotic threshold of HSCs is lowered by two TLR-4 polymorphisms that are protective against fibrosis[87].

The expression of chemokines and adhesion molecules in HSCs by TLR-4 signaling is likely also involved in macrophage recruitment to fibrogenesis sites[86].

LSECs and Kupffer cells play important roles in the clearance of gut-derived LPS without inducing local inflammatory reactions under physiological conditions. LPS tolerance in LSECs depends on reducing the nuclear translocation of NF-κB without a change in TRL-4/CD14 surface expression or scavenger activity[88]. The C-X-C chemokine receptor type-(CXCR)4 was recently demonstrated to be a part of the LPS "sensing apparatus", and inhibition of CXCR4 expression in endothelial cells (by RNA interference) decreased IL-6 production, LPS binding and chemotaxis[89]. CXCR4 over-expression on the LSECs membrane is driven by chronic injury[90,91], and CXCR4 expression likely plays a central role in provoking fibrosis after chronic insult. CXCR4 down-regulation (together with CXCR7 expression) stimulates regeneration immediately after injury. LSEC phenotype conversion from a CXCR7- to a CXCR4-expressing cell may enhance the response to gut-derived LPS, which provides a further mechanism for the induction of TLR-4 activation and pro-fibrogenic cascade.

Hepatic progenitor cells, which were traditionally not described as TLR-4-expressing elements, were also recently demonstrated to be involved in TLR-4 signaling. TLR-4 expression by hepatic progenitor cells and inflammatory cells at the porto-septal and interface level in patients with NAFLD, was supported by increased LPS activity and associated with the activation of fibrogenic cells and the degree of fibrosis[92]. Biliary cells of the interlobular bile ducts and liver progenitor cells exhibit the highest TLR-4 immunohistochemical expression in patients with chronic hepatitis C, which correlated with the degree of inflammation, portal/septal myofibroblasts activity and fibrosis stage[93].

Hepatic progenitor cells, which are bipotential stem cells that reside in human and animal livers, differentiate towards hepatocytic and cholangiocytic lineages, and proliferation leads to the so-called "ductular reaction"[93-96]. Studies in patients with biliary disorders and experimental models of biliary fibrosis demonstrated that the ductal epithelium expressed several profibrogenic and chemotactic proteins[97-100], and TLR-4 expression by biliary epithelial cells was associated with inflammation

and fibrosis progression[93,101,102]. Proinflammatory cytokines produced in response to TLR-4 signaling may participate in the cross-talk between hepatic progenitor cells and proliferating cholangiocytes or inflammatory cells and portal/septal myofibroblasts[93].

Increased TLR-4 expression by cholangiocytes represents a marker of loss of tolerance to LPS, which contributes to chronic biliary inflammation[102]. TLR-4-expressing cholangiocytes produce high levels of IL-1β, IL-8, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF-β[101]. LPS treatment of cultured biliary epithelial cells induces nuclear translocation of NF-κB, NF-κB-DNA binding and the production of TNF-α[103]. Human cholangiocytes cultured under normal physiological conditions express let-7i (a family members of let-7 miRNA), which post-transcriptionally downregulates TLR-4 expression[104]. The formation of an NF-κB p50-C/EBPβ silencer complex after LPS treatment or *Cryptosporidium parvum* infection inhibits the transcription of Let-7i and leads to increased TLR-4 expression[104,105]. This mechanism was hypothesized to allow detection and response to microbes without enhancing the inflammatory response.

Activation of the hepatic progenitor cell compartment and the consequent ductular reaction are also associated with the severity of nonbiliary chronic liver disease[93,106-108], and endotoxin also exhibits a role in stem cell/progenitor activation in other organs. LPS directly induces the proliferation of embryonic stem cells and adult tissue-specific stem cells/progenitors[109], hematopoietic progenitors[110], bone marrow mesenchymal stem cells[111]. The transplantation of p53-deficient hepatic progenitor cells transduced with TLR-4 results in liver-tumor development in mice following repetitive LPS injection[80].

CONCLUSION

The term "gut-liver axis" comes from the evidence of a strict interconnection between the gut and liver physiology and pathophysiology, and gut microbiota were recently claimed as a key mediator of this linkage. Chronic liver diseases are associated with qualitative and quantitative changes in the intestinal microbiota, which are partially dependent on the specific hepatic disease, and dysbiosis is almost always present during liver cirrhosis, regardless of the etiology of liver injury. Altered gut microflora contribute to intestinal dysmotility, inflammation and mucosal leakage. Finally, intestinal barrier damage allows the translocation of viable microorganisms and bacterial products, which reach the liver through portal blood and activate inflammatory pathways on liver cells.

These bases suggest that the TLR-4 receptor for bacterial endotoxin plays a starring role in the gut-liver axis. TLR-4 is activated in intestinal muscolaris macrophages, which are stimulated to produce and release prostaglandins and cytokines, and intestinal SMCs, which exhibit altered contractility with resulting dysmotility. TLR-4 activation in the gut exacerbates intestinal mucosal damage and bacterial translocation. Finally, most hepatic cell types express TLR-4, and LPS directly activates TLR-4 signaling in the liver once normal immune tolerance is exceeded. TLR-4 activation in Kupffer cells, HSCs, hepatocytes and cholangiocytes is implicated in most of inflammatory and fibrogenic pathways and activation contributes to the progression of liver disorders and complications of liver cirrhosis.

There are two promising strategies to hinder the deleterious effects of excessive TLR-4 activation: Modulation of gut microbiota to reduce the amount of TLR-4 ligand and direct interference with TLR-4 signaling. Drugs that are capable of attaining the first outcome, such as probiotics, prebiotics and antibiotics, already exist, and probiotic therapy produces beneficial effects on the liver, at least in the context of NAFLD[112]. Drugs of the second class are far from clinical application, but TLR-4 antagonism could weaken host immunity. However, some interesting evidence already comes from experimental studies, and the TLR-4 antagonist eritoran tetrasodium was recently demonstrated to attenuate liver damage in a liver ischemia/reperfusion injury model[113].

In conclusion, TLR-4 has emerged as a clear protagonist in the gut liver-axis over the past few years. Now that the pathophysiological basis is mostly known, it is time to see whether we can convert this knowledge into effective therapeutic interventions.

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EDITORIAL

New-found link between microbiota and obesity

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Abstract

Due to the grave pathological role of obesity, worldwide research is being continued to find out the causative factors involved in it. Recent advances in this field reveal a possible relationship between the compositional pattern of gut microbiota and genesis of obesity. Several study results have shown that short-chain fatty acids (SCFAs, microbiota-induced fermentation products) and lipopolysaccharides (LPS, an integral component of Gram negative microorganisms) play the key role in linking the two. Though several SCFAs are produced as microbiota-fermentation products, three of them, *i.e.*, butyrate, propionate and acetate have been found to be definitely involved in obesity; though individually they are neither purely obesogenic nor antiobesogenic. Out of these, butyrate and propionate are predominantly antiobesogenic. Butyrate, though a major energy source for colonocytes, has been found to increase mitochondrial activity, prevent metabolic endotoxemia, improve insulin sensitivity, possess anti-inflammatory potential, increase intestinal barrier function and protect against diet-induced obesity without causing hypophagia. Propionate has been found to inhibit cholesterol synthesis, thereby antagonizing the cholesterol increasing action of acetate, and to inhibit the expression of resistin in adipocytes. Moreover, both these SCFAs have been found to cause weight regulation through their stimulatory effect on anorexigenic gut hormones and to increase the synthesis of leptin. Unlike butyrate and propionate, acetate, which is substantially absorbed, shows more obesogenic potential, as it acts as a substrate for hepatic and adipocyte lipogenesis. High fat diet increases the absorption of LPS, which, in turn, has been found to be associated with metabolic endotoxemia and to induce inflammation resulting in obesity. Multiple independent and interrelated mechanisms have been found to be involved in such linking processes which are discussed in this review work along with some possible remedial measures for prevention of weight gain and obesity.

Key words: Microbiota; Obesity; Butyrate; Propionate; Acetate

Core tip: The objective of this article is to relate gastrointestinal microbiota with obesity positively. This idea itself is most innovative. In this article, probable mechanisms involved in relating microbiota with obesity

have been discussed. Its key findings are: (1) The gut microbiota play a definite role both in genesis and retardation of obesity; (2) Microbiota-derived lipopolysaccharides and short-chain fatty acids mediate the obesogenic action; (3) Fatty diet not only adds calories but also shifts microbiota compositional pattern in favour of obesity; and (4) The obesogenic actions are mediated through receptor activation, modification of cytokine and endocrine function and gene expression.

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INTRODUCTION

Obesity, in both males and females, was considered simply as a negative criterion while assessing beauty. But recently, in addition to its previous role, it is considered to be an important marker for several diseases; particularly, hypertension, type 2 diabetes mellitus (T2DM) and metabolic syndrome where it plays a definite and significant pathological role[1,2]. Multiple etiological factors have been attributed to the genesis of obesity, of which hereditary predisposition, wrong dietary habits (fatty food) and life-style (lack of exercise) are important[3]. Besides these, certain hormonal imbalances[4] and side-effects of some drugs[5] also contribute towards its development. But unfortunately there are many obese individuals, in whom, these causative factors fail to explain the cause of their obesity. Therefore, because of its grave pathological role, research is still going on to find out the factors other than the above-mentioned ones, so that a remedial measure can be taken to prevent the development as well as progression of this worldwide epidemic[6].

Recently, it has been observed that the composition of gut microbiota of healthy persons is different from that of obese T2DM patients. Such observations suggested a possible relationship between the compositional pattern of gut microbiota and pathology of metabolic disorders. Human colon harbours a vast number of microorganisms which are extremely diverse. Out of these, three phyla, *Bacteroidetes* (Gram negative), *Firmicutes* (Gram positive) and *Actinobacteria* (Gram positive), are most abundant and have been found to play a dominant role in the pathophysiology of metabolic disorders - specifically, obesity. Other phyla also contribute, but to a lesser degree[6]. All these colonic microbiota cause fermentation of nondigestible carbohydrates resulting in the formation of short-chain fatty acids (SCFAs) along with gases like CO_2 and H_2[7]. It has been shown that acetate and propionate are mainly produced by the phylum *Bacteroidetes*, whereas butyrate is the predominant product of the phylum *Firmicutes*[6]. Of these SCFAs, butyrate mainly serves as an energy source for colonic epithelium[8,9], whereas propionate, getting absorbed through portal circulation, takes part in gluconeogenesis[8]. Acetate, on the other hand, reaches peripheral tissues after absorption through systemic circulation where it acts as a substrate for synthesis of cholesterol[8,10,11]. Butyrate, besides being an energy source for colonocytes, has been found to increase insulin sensitivity (in mice)[9], possesses obesity-related antiinflammatory action (in humans)[12], can give protection against diet-induced obesity without causing hypophagia[13], may protect against colon carcinoma[8,10], and increase the leptin gene expression[11]. Propionate, in addition to contributing towards gluconeogenesis, also reduces the intake of food[13] and cholesterol synthesis[11] along with a favorable effect on leptin gene expression[11]. Acetate, in addition to serving as a substrate for synthesis of cholesterol, also takes part in the *de novo* synthesis of lipids in liver[14]. Because of the above-mentioned functions of the microbiota-derived SCFAs, which appear to be closely related to obesity, both adversely as well as beneficially, an attempt has been made to review the work-results of several prominent investigators in this field, which may shed a light on the justification of "linking microbiota to obesity".

MICROBIOTA IN NORMAL GUT AND OBESITY

Microbiota in normal gut

The gut harbours the greatest density of microorganisms in the body (*e.g.,* about up to 1.5 kg of bacteria in the human gut) with *Firmicutes*, *Bacteriodetes* and *Actinobacteria* constituting the dominant phyla[7,15,16]. Generally, *Firmicutes* and *Bacteroidetes* are most abundant, followed by *Proteobacteria* and *Actinobacteria* with minor contributors like *Verrucomicrobia* and *Fusobacteria*[16].

Faecalibacterium prausnitzii (*F. prausnitzii*) is the most abundant bacterium in the human intestinal microbiota of healthy adults (Table 1). It represents more than 5% of the total bacterial population. *F. prausnitzii* species is a major representative of *Firmicutes* phylum, *Clostridium* class, *Ruminococcaceae* family[17]. While the *Bacteroidetes* phylum mainly produces acetate and propionate, the *Firmicutes* phylum has butyrate as its primary metabolic end product[7].

Microbiota in obesity

Gut microbiota have been found to be significantly changed in humans and animal models of obesity, comprising a decrease in bacterial diversity[15,18] as well as composition, such as a reduced abundance of *Bacteroidetes* with a proportional increase in *Firmicutes* phylum[6,9,18-21].

In obese animals, Ley *et al*[22] found a difference in the ratio of *Bacteroidetes* and *Firmicutes*, where the obese mice displayed a decrease in *Bacteroidetes* with a corresponding increase in *Firmicutes* in comparison to their counterparts. In agreement with the results from

Table 1 Prevalence of gut microbiota in health and disease

Microbiota in normal gut	Microbiota in obesity
Firmicutes phylum	Increase in *Firmicutes* phylum
Bacteriodetes phylum	Reduced abundance of *Bacteroidetes*
Actinobacteria phylum	A higher level of *Actinobacteria* phylum
Verrucomicrobia phylum	Lower proportion of *Verrucomicrobia*
Faecalibacterium prausnitzii species	Reduced abundance of *Faecalibacterium prausnitzii* species

animal studies, it seems that human obesity is linked with a reduced abundance of intestinal *Bacteroidetes* associated with a high abundance of *Firmicutes*. However, these results have been contradicted by other studies[11]. Studies of Duncan *et al*[23] did not show any difference in the proportions of *Bacteroidetes* and *Firmicutes* in the feces of lean and obese subjects. In another investigation, overweight and obese subjects had a ratio of *Bacteroidetes* to *Firmicutes* in favour of *Bacteroidetes*. Moreover, many authors have shown no change or even an increase of *Bacteroidetes* in overweight[6]. Besides these two phyla, a higher level of *Actinobacteria* has been demonstrated in obese persons[24]. On the other hand, Clarke *et al*[25] reported that the gut microbiota of obese individuals contained a lower proportion *Verrucomicrobia*, *i.e.,* abundance of this phylum in the gut is reduced in obese persons (Table 1). From these observations, it appears that the phylum level difference of the gut microbiota between obese and lean individuals may not be universally true[11]. But overall analysis of results point towards an increase in *Firmicutes*[6].

Methane-producing *Archaea*, a domain of single-celled microorganism, have been found to be present in greater abundance in obese mice and humans compared with lean subjects. Recently, in an investigation, germ-free mice were colonized with *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) (an adaptive bacterial forager of dietary polysaccharides) alone or either with *Methanobrevibacter smithii* (*M. smithii*) or the sulfate-reducing bacterium *Desulfovibrio piger* (*D. piger*). The results showed that cocolonization with *M. smithii* but not *D. piger,* induced *B. thetaiotaomicron* to ferment dietary fructans to acetate, resulting in a significant increase in host adiposity compared with monocolonized or *B. thetaiotaomicron*/*D. piger* cocolonized mice[20].

In an investigation, the numbers of hydrogen-producing *Prevotellaceae,* a family in the phylum *Bacteroidetes*, and *Archaea*, represented primarily by members of the order *Methanobacteriales* (hydrogen-oxidizing methanogens), were at a higher level in obese individuals compared with lean subjects and with those after gastric bypass. The investigators hypothesized that hydrogen transfer between bacterial and archaeal species may raise energy uptake by the large intestine in obese individuals *via* methanogens removing fermentation intermediates, such as H_2 or formate, thus relieving thermodynamic limitations and allowing greater production of SCFAs that are then available to be absorbed across the intestinal epithelium[20]. On the contrary, Schwiertz *et al*[26] found no difference in the abundance of *Archaea* in overweight or obese humans, which brings into question the usefulness of *Archaea* as a potential biomarker of obesity.

The intestines of obese humans and mice have been found to be enriched with *Erysipelotrichi*, a class of bacteria within the phylum *Firmicutes*, and *Clostridium ramosum* (*C. ramosum*), a member of the *Erysipelotrichi*, is found to be linked with symptoms of the metabolic syndrome in humans. Thus, Woting *et al*[27] speculated that *C. ramosum* promotes obesity and related pathologies.

Obese children were found to display an elevated *Firmicutes*-to-*Bacteroidetes* ratio compared with their lean counterparts. Furthermore, low relative proportions of *Bacteroides vulgatus* and high concentrations of *Lactobacillus* spp. were found in the obese children and were positively correlated with plasma high-sensitivity C-reactive protein[21]. Million *et al*[28] have shown that *Lactobacillus reuteri* was linked with obesity in adults. These results thus indicate a possible role of *Lactobacillus* species in body weight and obesity. Moreover, *Staphylococcus* spp. were found to be positively linked with energy intake in all children[21].

Obese-prone (OP) donor and germ-free recipient animals have been found to harbour specific species from *Oscillibacter* and *Clostridium* clusters XIVa and IV, which were totally absent from their obese-resistant counterparts. Indeed, Duca *et al*[18] have reported high levels of bacteria from the *Ruminococcus* genus in OP rats, similar to that found in obese humans and high fat-fed mice. It is known that *Ruminococcus* is phylogenetically heterogenous, and most of its species fall under several *Clostridium* clusters, including *Clostridium* clusters IV and XIVa. But peculiarly, *Clostridium leptum* (cluster IV) has been found to be associated with both obesity and weight loss (Table 2). From the above discussion, it may be mentioned that unfavourable microbiome seems to be a predisposing factor for development of obesity.

While some gut bacteria groups correlated with energy intake, obesity, and metabolic changes, others, such as *F. prausnitzii*, linked with alteration in the inflammatory state and diabetes[29]. The presence of *F. prausnitzii* species is directly associated with the reduction in low-grade inflammation state in obesity and diabetes (independently of calorie intake)[17,29] (Table 1).

SCFAs

It is well established that the human intestine harbours a vast number of microorganisms, known as gut microbiota, whose metabolic end products (mainly SCFAs) interfere with the absorption of digestion end products as well as energy homeostasis of the host[19].

In intestine, the sites of production of SCFAs are distal small intestine and colon where nondigestible carbohydrates like resistant starch, dietary fiber, and other low-digestible polysaccharides are fermented

Table 2 Microbiota having doubtful role in obesity

Microbiota
Archaea (a domain of microorganisms)
Phylum *Firmicutes*: *Erysipelotrichi* (a class of bacteria)
Methanobacteriales (an order of bacteria)
Prevotellaceae (a family of bacteria)
Ruminococcus (a genus of bacteria)
Bacteroides thetaiotaomicron and *Methanobrevibacter smithii*
Clostridium ramosum (a member of the *Erysipelotrichi*)
Clustridium leptum (cluster Ⅳ) (associated with both obesity and weight loss)
Specific species from *Oscillibacter* and *Clostridium* clusters XⅣa and Ⅳ
Lactobacillus spp. - *Lactobacillus reuteri*

by the saccharolytic bacteria which include the phyla *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Acetate and propionate are the main products of *Bacteroidetes* phylum and butyrate is mainly produced by *Firmicutes* phylum. Most bacterial activity is found in the proximal colon where substrate availability is the highest. But towards the distal colon, the availability of substrate decreases, and the extraction of free water lowers the diffusion of substrates and microbial products. This makes the proximal colon to be the principal site of fermentation, where, mainly nondigestible carbohydrates are fermented by saccharolytic bacteria, primary fermenters being *Bacteroidetes* and the main fermentation products are SCFAs together with gases like CO_2 and H_2[7]. Of the three SCFAs, butyrate is practically considered as a favourable marker (antiobesity) of obesity and its amount of production is determined by the composition of microbiota, population of the microorganisms producing it and the pH of the large intestine. Change in substrate bioavailability can alter the composition of butyrate-producing bacterial population and thus affect butyrate production[8]. It has been demonstrated that when the human fecal pH is 5.5; butyrate producing bacterial population (*Firmicutes* phylum) comprises 20% of the total bacterial population. But in the distal parts of large intestine, where fermentable dietary fiber availability is limited, the luminal pH is raised to 6.5. At this site, not only the bacteria producing butyrate, practically disappear completely, but also there occurs a significant increase in the population of acetate- and propionate-producing bacteria, whose products are mainly obesogenic[7].

An analysis of the population data regarding the production of SCFAs in proximal and distal colon shows that the production is in the order of acetate > propionate > butyrate. When calculated in a molar ratio, it was found to be 60:20:20 or 3:1:1, respectively[10]. It has been observed that out of the total SCFAs present in the colon, 90%-95% are constituted by acetate, propionate and butyrate together and their intraluminal individual concentrations have been found to be acetate 60%, propionate 25% and butyrate 15%[30].

After being produced in the colon, the above-mentioned three SCFAs are absorbed through gut epithelial cells but follow different patterns of absorption, distribution, metabolism and function. A substantial part of acetate is readily absorbed, reaches liver *via* portal circulation and subsequently, distributed throughout the whole body where it serves as a substrate for synthesis of cholesterol[11,13]. Because of the substantial absorption, plasma concentration of acetate is much more than the other two[30] and a small amount is available in the colon to be metabolized[10].

Propionate, like acetate, also reaches liver *via* portal circulation after absorption; but because of its primary utilization in gluconeogenesis (in the liver), its plasma concentration is less than that of acetate[10,11,30]. Butyrate, on the other hand, undergoes limited reabsorption, because it is primarily oxidized by the colonocytes and serves as a major source of energy for them[8,9,30].

It seems essential to mention here that absorption of these SCFAs through colonic epithelial cells alters the pH of colon, which in turn has an important influence on the composition and population of gut microbiota. It is so, because most of the SCFAs are absorbed in the colon being exchanged with bicarbonate and hence, the resultant luminal pH is determined by the rate of SCFA production by microbiota and the neutralizing capability of the bicarbonate. Due to its continuous absorption, decline in SCFA concentration from proximal to distal colon leads to a corresponding increase in pH from cecum to rectum. It has been demonstrated in animal and human fecal studies that gut pH has an important effect on the growth and composition of gut microbiota. Low luminal pH from ileum to cecum due to higher SCFA concentration, prevents the overgrowth of pH-sensitive pathogenic bacteria (like *Enterobacteriaceae* and *Clostridia*) and at pH 5.5, butyrate producing bacteria (*Firmicutes* phylum) comprise 20% of the total population (mentioned earlier). But as the luminal pH increases to 6.5 in more distal colonic sites due to less production of SCFAs (as fermentable dietary fibers are less available here) and their absorption in exchange with bicarbonate, the butyrate producing bacteria practically disappear along with a concomitant rise in acetate and propionate-producing bacteria (*Bacteroidetes* phylum)[7].

A detailed discussion has been made above about the multiple bacterial phyla producing several metabolites, of which three SCFAs play a dominant role in the development, progression as well as retardation of obesity. These three SCFAs are butyrate, propionate and acetate, produced during the fermentation of complex dietary carbohydrates (polysaccharides and oligosaccharides), proteins, peptides, and glycoprotein precursors by the microbiota in the colon and distal small intestine[10,11,13]. Chemically, SCFAs are saturated aliphatic organic acids containing one to six carbons (Acetate C2, propionate C3 and butyrate C4)[7].

FACTORS CONTRIBUTING TOWARDS GENESIS OF OBESITY

Besides the well known and established causes of

obesity like genetic predisposition, excessive intake of high calorigenic diet (fatty food) and lack of exercise[3] which favours storage of calorie in the form of fat in adipocytes, recently researchers in the field have shown the contribution and involvement of several other factors, like hormonal imbalance[4]; inflammatory cytokines of adipocyte and nonadipocyte origin; adipocytokines like adiponectin[31], leptin[32], and resistin[33], *etc*., toll-like receptors (TLR)[34] and many others in the genesis of obesity[33].

In addition to these, multiple study results have shown a close link between the compositional patterns of "intestinal microbiota" and "obesity"- the microbiota affecting the above - mentioned obesogenic factors through several mechanisms. A detailed account of the microbiota with their composition and population ratio and their metabolic end products (particularly SCFAs), have already been discussed. Here, an attempt has been made to discuss the various mechanisms involved in their obesogenic as well as antiobesity activity, although some of the observations appear to be controversial and inconclusive.

Though intestinal microflora comprises several phyla of microorganisms, focus has been made on three phyla, namely *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. These three phyla generate multiple metabolites out of which three SCFAs - butyrate, acetate and propionate have been shown to be definitely related with obesity. It may be mentioned in the beginning that none of these bacterial phyla is purely obesogenic or antiobesogenic. This is so, because individually they produce more than one SCFA, each of which possessing opposite actions as metabolites, which in turn possesses both the actions[7].

For this reason, while evaluating their obesogenic or the antiobesogenic potency, instead of taking the population of a single bacterial phylum, the population ratio of more than one phylum has been taken into consideration[6,7,19,21]. Several metabolic studies have suggested that imbalances in the intestinal bacterial population may result in obesity, systemic inflammation and metabolic dysfunction[14,35].

Gut microflora are involved in obesity through some of their constitutive structural materials and through some of their metabolic end products (SCFAs). Therefore, the mechanisms by which they contribute towards the development of obesity may be discussed under two headings: (1) The role of lipopolysaccharide (LPS) which is a structural component of bacteria; and (2) the role of SCFAs which are produced as bacterial metabolites of dietary compounds[11,14].

Role of LPS

Recently, it has been shown that obesity is associated with a chronic and systemic low-grade inflammation which is due to an innate immune response to LPS. It is an intrinsic constituent of Gram negative bacterial cell wall. It is considered as an endotoxin and found at low concentrations in the blood of healthy persons. But substantially high concentrations of LPS have been demonstrated in obese individuals, where the obesity is diet-induced and has a genetic predisposition. High fat diet, both in animals and humans, has been found to alter the gut microbiota composition (more in favour of Gram negative phylum), which in turn increases the production and intestinal permeability of LPS, resulting in its high plasma concentration and development of "metabolic endotoxemia"[20]. Cani *et al*[36] have found that compositional pattern of microbiota, induced by a high-fat diet, could increase gut permeability which is an important hallmark of endotoxemia. Such microbiota were found to reduce the expression of host genes which code for the intestinal tight junction proteins like ZO-1 and occludin - necessary for normal gastrointestinal permeability character. Such microbiota-induced altered gastrointestinal epithelial integrity could result in intestinal absorption of the whole bacteria along with their products. It has been observed that in mice, taking a high-fat, such bacterial absorption is higher than those taking a standard chow and was found to be reversed by administering an appropriate probiotic bacterium[37].

LPS has been found to induce inflammation resulting in development of obesity. In a comparative study, it has been shown that when low doses of LPS were administered to mice for 4 wk, they developed obesity similar to 4 wk of a high-fat diet. LPS-induced inflammatory reactions are mediated through an immunoprotein called cluster of differentiation (CD) 14. When LPS was administered through $CD14^{-/-}$ rats, there was no weight gain. It is interesting to note that high fat diet is not only directly responsible for obesity but also indirectly aggravates it by increasing the absorption of endotoxin LPS *via* lymph by integrating it to chylomicrons. As high fat diet in humans increases the formation of chylomicron, more chylomicron is available to be integrated with LPS and hence, more absorption of this endotoxin in comparison to low fat diet. Mice develop endotoxemia when they consume high fat diet. Studies have shown that when such mice were treated with ampicillin and neomycin, endotoxemia was found to be reduced because of the antimicrobial-induced altered gastrointestinal microbiota. High plasma concentration of LPS has been found to be associated with increased levels of CD14 and interleukin-6 (IL-6) - the markers of inflammation. Because of these observations it may be inferred that regular intake of high fat diet, increases LPS absorption into systemic circulation, resulting in LPS-induced inflammation and obesity[37].

Chronic low-grade inflammation found in endotoxemia has been demonstrated to be due to activation of TLR-4 by LPS and dietary saturated fatty acids. TLR-4 activation induces upregulation of common intracellular inflammatory pathways like c-Jun N-terminal kinase and nuclear factor-kappa B in adipocytes and macrophages resulting in development of insulin resistance and increased adiposity[6]. Mice, lacking TLR-4, have been found to be resistant to diet-induced obesity and insulin resistant[37].

de La Serre *et al*[38] have demonstrated that high-fat

diet not only alters the composition of gut microbiota, but also causes increased activation of intestinal TLR-4. Such receptor activation results in gastrointestinal inflammation which in turn induces hyperphagia and thus, makes the animal an obese phenotype.

A neural mechanism has been suggested to explain LPS-induced obesity, in which the vagal afferents of diet-induced obese rats are found to be leptin resistant, and thus, develop hyperphagia and weight gain, which in turn, lead to increased food (fat) intake and LPS production, thereby increasing obesity and aggravating the inflammation further[37].

As mentioned earlier, LPS, which induces inflammation and increases adiposity resulting in obesity, is known to be a Gram negative bacterial product. But, there are confusing observations, where obese persons have more *Firmicutes* (Gram positive) and less *Bacteroidetes* (Gram negative) than lean individuals. Inspite of such confusions, recent observations show that obese person's microbiota are rich in *Prevotellaceae* (a subgroup of *Bacteroidetes*), which is a good source of LPS[37].

Though microbiota-constituent LPS is proinflammatory, some microbiota metabolite SCFAs possess immunoregulatory property and reduce inflammation. Studies have shown butyrate to have antiinflammatory action through inhibition of lymphocyte proliferation, and IL-2 and interferon-γ production. On the other hand, acetate and propionate increase interferon-γ level. The resultant effect of these three SCFAs is immunoregulatory[37].

Role of SCFAs

It has already been mentioned about the production of three SCFAs by different phyla of gastrointestinal microflora[7] and the obesogenic as well as antiobesogenic property of individual SCFAs which make it difficult to categories each of them as purely obesogenic or antiobesogenic. Of course, a broad characterization can be made where acetate appears to be predominantly obesogenic, whereas butyrate and propionate are mainly antiobesogenic[8-13,39].

Interesting and novel mechanisms have been found to be involved in the causation and prevention of obesity by the above-mentioned three SCFAs. It may be convenient to mention the contribution of individual SCFAs towards the genesis as well as prevention of obesity and subsequently, discuss the underlying mechanisms involved in such actions: (1) Butyrate has been found to be a major energy source for colonocytes[8-11,13,30]. In the colonocyte-mitochondria 70% to 90% of the SCFA (butyrate)[10] is oxidized into acetyl-CoA, which is subsequently processed through tricarboxylic acid cycle to generate large quantity of ATP[8]. It has been shown that in addition to producing butyrate, the butyrate-producing microbes also increase the expression of the enzymes taking part in the colonocyte-mitochondrial SCFA-oxidative reactions[9]; (2) Besides supplying energy, butyrate also has a controlling role over the mechanisms involved in cellular apoptosis, proliferation and differentiation[10]; (3) Butyrate has been shown to possess some mixed metabolic effects which include an increase in mitochondrial activity, prevention of metabolic endotoxemia and activation of intestinal gluconeogenesis. These actions are mediated through gene expression and regulation of hormonal activity[9]; (4) Butyrate, when given orally to mice, has been found to improve insulin sensitivity and increase energy expenditure by improving mitochondrial function which may result in reduction of obesity[9]; (5) Some studies have indicated the antiinflammatory potential of butyrate which may contribute towards a decrease in obesity-associated metabolic complication, because of its capability to increase intestinal barrier function[12]. These effects of butyrate support the observation that decreased population of intestinal butyrate producing bacteria is associated with metabolic risk in humans; (6) Butyrate has been found to be protective against diet-induced obesity without causing hypophagia. Acetate which is considered as obesogenic, also possesses this beneficial function like butyrate[13]; (7) Butyrate and propionate (beneficial SCFAs) cause weight regulation at least partially by controlling food intake; the action appears to be mediated through their stimulatory effect on the anorexigenic gut hormones. It may be mentioned here that acetate also inhibits weight gain, but through mechanisms which are independent of suppression of food intake and acute gut hormone effect[13]; (8) Xiong *et al*[40] had demonstrated the potential of butyrate and propionate to increase the expression of the gene coding for synthesis of leptin (Table 3); and (9) Besides these antiobesogenic properties, both butyrate and propionate have been shown to possess a definite protective role against colon carcinogenesis[8,10].

Like butyrate, propionate also possesses favourable some effects in obesity. They are as follows: (1) The SCFA has been found to reduce food intake and regulate body weight, similar to butyrate[13]; (2) It decreases cholesterol synthesis by inhibiting the activity of the enzyme acetyl-CoA synthetase (the enzyme converts acetate to acetyl-CoA), thereby antagonizing the cholesterol increasing action of acetate[10,11]; (3) Moreover, propionate has been found to be a precursor for gluconeogenesis in the liver[10,14]. This may decrease the hepatic synthesis of cholesterol because fatty acids necessary for cholesterol synthesis are diverted towards synthesis of glucose (gluconeogenesis)[14]; (4) It has been shown that like butyrate, propionate also stimulates the formation of the anorexigenic hormone leptin[40] (Table 3); and (5) However, propionate inhibits the expression of resistin in human adipose tissue[39].

Of all the three SCFAs, acetate seems to be more obesogenic than butyrate and propionate because: (1) It is a substrate for lipogenesis[8,14] and cholesterol synthesis in liver and other tissues[8,11]. This SCFA is readily and substantially absorbed by the colonocytes and though,

Table 3 Gross mechanisms involved in short-chain fatty acids-induced obesity

Butyrate
A major energy source for colonocytes
Involved in cellular apoptosis, proliferation and differentiation
Possesses metabolic effects like increase in mitochondrial activity, prevention of metabolic endotoxemia and activation of intestinal gluconeogenesis
Improves insulin sensitivity and increases energy expenditure by improving mitochondrial function resulting in reduction of obesity
Increases intestinal barrier function - an antiinflammatory potential
Protects against diet-induced obesity without causing hypophagia - the action being mediated through stimulation of anorexigenic gut hormones
Increases the expression of the gene coding for synthesis of leptin
Propionate
Increases the expression of the gene coding for synthesis of leptin
Protects against diet-induced obesity without causing hypophagia - the mechanism being similar to butyrate
Decreases cholesterol synthesis by inhibiting the activity of the enzyme acetyl-CoA synthetase
Acts as a precursor for hepatic gluconeogenesis thereby decreasing the hepatic synthesis of cholesterol
Inhibits the expression of resistin in human adipose tissue
Acetate
Acts as a substrate for lipogenesis and cholesterol synthesis in liver and other tissues
Gives protection against diet-induced obesity without causing hypophagia

some part of it is utilized in the liver for lipogenesis, a significant amount reaches systemic circulation and is delivered to the peripheral tissues[13] for synthesis of cholesterol (specifically in adipose tissues and mammary glands, whose cytosol contains acetyl-CoA synthetase, the enzyme essential for utilization of acetate for lipogenesis)[10]. Human studies have shown that when lactulose (synthetic nonabsorbable sugar, metabolized by microbiota to produce high amounts of acetate) was administered to the diets of six volunteers for two weeks, there was a significant increase in both total and low-density lipoprotein cholesterol, apolipoprotein B and plasma concentration of acetate in comparison to the control group[11]; and (2) Though predominantly obesogenic, some workers have demonstrated the obesity-protecting role of acetate, which is less than that of butyrate and propionate. Like butyrate, it gives protection against diet-induced obesity without causing hypophagia and thus, the action is independent of suppression of food intake and does not have any acute effect on gut hormones[13] (Table 3). It has been demonstrated that acetate increases cholesterol synthesis and propionate, though regulates it, does not affect serum cholesterol levels[8,11] and is primarily utilized for gluconeogenesis[8,10,14]. But when the two SCFAs are administered simultaneously, serum cholesterol level does not rise[11]. This may be due to increased gluconeogenesis by propionate consuming more fatty acids and thus diverting them from getting utilized by acetate for synthesis of cholesterol[14]. Therefore, though acetate increases fatty acid synthesis, they take part in gluconeogenesis rather than being used for synthesis of cholesterol and thus, plasma level of cholesterol does not rise[10,11].

It has been demonstrated that fecal concentration of SCFAs are 20% higher in obese individuals than their lean counterparts. But such higher SCFAs concentration in feces may reflect a compensatory protective mechanism against obesity, in which a greater amount is eliminated from the increased amount of SCFAs produced, thereby preventing increased accumulation of SCFA in the intestinal lumen for obesogenic action[37].

MECHANISM OF ACTION OF SCFAS AT THE MOLECULAR LEVEL

Some important actions of these three SCFAs have been found to be mediated through activation of endogenous free fatty acid receptor (FFAR) like FFAR2 and FFAR3 which are otherwise designated as Gpr43 and Gpr41, respectively, because they belong to G-protein coupled receptor family of receptors[13,37]. Presence of both these receptors has been demonstrated in adipocytes, epithelial cells and enteroendocrine cells. Activation of these two receptors leads to an increase in expression of satiety hormone polypeptide YY (PYY) and increase in intestinal motility. In addition to the above effect, Gpr41 activation also increases the expression of leptin in adipocytes. It has been observed that when SCFA-producing bacteria were administered to germ-free mice, the mice gained weight along with an increase in body fat. But, mice (both germ-free and conventional), deficient in Gpr41 did not show such effects. Such observation indicates that weight gain occurs through activation of Gpr41[37]. Moreover, Samuel *et al*[41] have shown that the expression of PYY in the above-mentioned mice was lower in the mice with intact Gpr41. Reduced production of PYY leads to decreased gut motility and hence, decreased dietary energy harvest[19]. Besides increasing leptin expression in adipocytes, Gpr41 activation also increases hepatic lipogenesis. Hence, this receptor is considered as a probable regulator of energy balance of the host[37].

SCFAs, like butyrate and propionate, increase the formation of the gut hormone glucagon-like peptide-1 (GLP-1). It reduces food intake by decreasing appetite. Maximal induction of GLP-1 requires activation of Gpr41, but is not essential[13].

Nondigestible carbohydrates (NDC) are known to be antiobesogenic because they are not digested in the intestine but are fermented in the large bowel resulting in the formation of SCFAs. Ultimately, they (SCFAs) mediate some of the antiobesogenic actions of NDC.

Propionate stimulates Gpr43 in caloric enteroendocrine cells leading to increased release of PYY and GLP-1 (anorexigenic gut hormones). It also activates Gpr43 in adipocytes, which reduces output of FFAs into circulation and thus, it results in increased insulin sensitivity. Hence, the formation of more propionate in the colon, by consuming NDC, may be beneficial in obesity[42].

However, food rich in fermentable fibers are seemed to stimulate obesity through harvested energy by their SCFAs (metabolites). But epidemiological study results suggest that they prevent it rather than promoting. It may be explained by the fact that these SCFAs, by stimulating FFARs, cause satiety *via* increased production of GLP-1 and PYY[18]. Thus, they are not obesogenic[14,18].

Certain study results have shown that mirobiota-derived SCFAs modulate (increase) the secretion and gene expression of *GLP-1* and *PYY* which are known to be satiety hormones[18,37]. Fasting-induced adipocyte factor (Fiaf) has been found to suppress the production of adipocyte-LPL (hormone sensitive lipase) which leads to an increase in lipolysis of triglycerides in adipocytes and modulation of fatty acid oxidation in adipocytes and skeletal muscles. It has been shown that physiological appetite regulators regulate the expression of Fiaf in the hypothalamus and exert their anorexigenic effect through inhibition of hypothalamic AMP-activated protein kinase (AMPK) activity. This suggests a central regulatory role of Fiaf in energy metabolism[43].

Investigations on germ-free and conventionalized mice have shown that one of the mechanisms of energy harvest and adipocyte hypertrophy by microbiota is through inhibition of enterocyte Fiaf, leading to suppression of the actions of intestinal LPL and increased activity of PYY[19,44].

Metabolic degradation of a given source of energy is more with *Firmicutes* than with *Bacteroidetes*, resulting in increased absorption of calories and hence more weight gain[45]. Increased population of *Firmicutes* has been found to raise the number of lipid droplets, thereby proportionately intensifying fatty acid absorption[46]. Such a finding seems to involve several mechanisms. Microbiota may increase the metabolism of the host along with modification and increase in bile salt production. It favours more fatty acid (FA) absorption and hence, increased bioavailability[47]. In addition, intestinal microbes may directly prevent the lipolytic activities of the host[48]. They may indirectly change the physiological responses in the gut of the host, resulting in increased absorption. Finally, microbes may lower the rate of FA oxidation, which increases FA absorption[46]. In addition to these, *Firmicutes*-induced increased FA absorption may involve other specific mechanisms[45].

Methanogen, like *M. smithii* is found in 70% of human beings. It generates methane through anaerobic fermentation. It has been found to enhance the fermentation of polysaccharides and other carbohydrates by removing hydrogen atoms, leading to greater production of SCFAs and hence, their increased absorption. These SCFAs function as an extra source of energy which contributes towards weight gain and subsequent obesity[49].

Some gastrointestinal microbiota-components have been found to suppress the expression of the host genes which code for the synthesis of intestinal epithelial tight junction proteins and Fiaf, leading to increased adipocyte lipoprotein lipase (LPL) activity and hence, increased storage of liver-derived triglyceride in host fat cells and weight gain[22,37,43]. Interesting experiments on mice has been conducted to demonstrate the combined effect of microbiota and diet resulting in development of obesity. When mice reared in germ-free environment (hence absence of gastro-intestinal microbiota) were fed with a western-style diet (high fat, high sugar), they did not gain weight as compared with colonized mice with similar diet. This may be due to suppression of microbiota-induced gene expression and hence, inhibition of Fiaf formation resulting in increased fat metabolism, lower fat storage and decreased sugar absorption. Such altered lipid metabolism and storage is supported by the fact that germ-free mice were having higher levels of Fiaf and hence, lower LPL activity, higher muscle and hepatic levels of the key enzyme (phosphorylated AMPK) necessary for β-oxidation and lesser monosaccharide absorption from the intestine in comparison with colonised mice[37]. Thus, gut microbiota may be considered as an important environmental factor increasing dietary energy harvest and energy storage in the host[19]. But such observations may not be taken conclusive, because another study has demonstrated that germ-free mice significantly gained weight with western-style diet[37].

It has been shown that in the mucosa of small intestine of gnotobiotic mice, who harbour intestinal *C. ramosum,* there is upregulation of Glut2 and CD36 transcription. It suggests that this organism is responsible for more gain in body fat by an increase in intestinal absorption of glucose and lipid[27].

It may be mentioned here that though bacterial product LPS disrupts normal gastrointestinal integrity, bacterial SCFA metabolites acetate and butyrate strengthen it by increasing the secretion of mucin-2 (MUC-2) - the mucus secreted by goblet cells, which plays an important role to maintain healthy intestinal epithelial barrier. It has been shown that butyrate, when added to goblet cell lines, increased the secretion of MUC-2 23-fold and, thus, considered as a protective SCFA against intestinal translocation of bacteria and their products[37].

As mentioned earlier, acetate is known to be obesogenic because of its peripheral action. However, it has been shown that it can also control weight gain through its central action, where it produces an anorexigenic signal in the hypothalamic arcuate nucleus, through increased generation of gamma-aminobutyric acid (GABA), by augmenting the glutamate-glutamine (transcellular) cycle involved in GABA production[50].

CONCLUSION

The beneficial role of gastrointestinal microbiota for maintenance of proper health of the host is well

established. From the above discussion, it seems that out of the millions of species harbouring the gastrointestinal tract, only a few are linked with the genesis of obesity. Moreover, individual species of these is not harmful entirely; each of them possessing obesogenic as well as antiobesogenic property, for which, ratio of two species (like *Firmicutes* and *Bacteroidetes*) are taken into consideration when grouping them into harmful or beneficial group. Several researchers have observed that it is the dietary habit (fatty food) of the host which alters the population and composition of the microbiome, thereby shifting the ratio of the concerned pair in favour of obesity. Hence, by altering the nature of the diet (less fat and more NDC), an individual, in addition to reducing the total calorie intake, may also be able to shift the ratio in the opposite direction (antiobesity).

As one of the causes of obesity has been attributed due to the structural components (LPS) and metabolic end products (SCFAs) of certain gastrointestinal microorganisms, it is not wrong to consider obesity (at least partially, if not fully) as an infectious disease. Further research in this respect is needed to confirm this possibility and to find out selective chemotherapeutic agents, which will reduce or abolish the more harmful bacterial population. Another possible mechanism, which can cause weight loss or decrease obesity, is to implant the useful bacterial species in appropriate ratio.

Probiotics and prebiotics are known to alter the compositional pattern and population of gastrointestinal microflora and are used to prevent or ameliorate some of the antimicrobial chemotherapy-induced gastrointestinal side effects and some other gastrointestinal diseases. Because of the new found link between these microflora and obesity (both obesogenic and antiobesogenic), pharmaceutical industries may focus more on manufacturing the required pre- and probiotics which may be beneficial to counter this worldwide epidemic and its complications.

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TOPIC HIGHLIGHT

2015 Advances in Irritable Bowel Syndrome

Psychosocial impact of irritable bowel syndrome: A brief review

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Abstract

Irritable bowel syndrome (IBS) is a common disorder of the gastrointestinal tract with unclear etiology and no reliable biomarker. Like other chronic and functional disorders, medical treatments for IBS are suboptimal and the overall illness burden is high. Patients with IBS report high rates of psychopathology, low quality of life, and increased suicidal ideation. These patients also miss more days of work, are less productive at work, and use many healthcare resources. However, little is known about the burden of IBS on daily functioning. The primary aim of this paper is to review the current literature on the burden of IBS and to highlight the need for further research to evaluate the impact of IBS on daily activities. This research would contribute to our existing understanding of the impact of IBS on overall quality of life and well-being.

Key words: Irritable bowel syndrome; Quality of life; Biopsychosocial; Burden of illness; Daily activities

Core tip: Little is known about the burden of irritable bowel syndrome (IBS) on daily functioning. The primary aim of this paper is to review the current literature on the overall burden of IBS.

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IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) affects between 10%-15% of the population in North America with a 2:1 ratio of women to men[1,2]. It is classified as a functional gastrointestinal disorder, meaning that its symptoms are not associated with any structural or biochemical abnormalities in the gut. Symptoms of IBS are characterized

by abdominal pain and/or discomfort associated with diarrhea, constipation, or a mixture of both[3].

Although there are not currently any biological markers for IBS, recent research has identified physiological factors that contribute to the expression of IBS symptoms. One of the most commonly studied features of IBS is known as the brain-gut axis, which refers to neural and hormonal signaling between the central nervous system and the gastrointestinal tract. In the past decade, the pathophysiology of IBS has been attributed to dysregulation of the brain-gut axis primarily through a process known as visceral hypersensitivity (amplified pain signals originating in the neurons of the gut). In IBS patients, visceral hypersensitivity is believed to cause increased pain and stress symptoms in response to normal bowel activity, resulting in lower thresholds for colonic discomfort when compared to healthy controls[4,5].

Current medical treatments for IBS are relatively ineffective and do not address visceral hypersensitivity. Most available treatments are targeted at reducing specific IBS symptoms, especially symptoms associated with abnormal gut motility. These include laxatives, antidiarrheals, probiotics, antidepressants, and psychological interventions to enhance stress-management skills and to help patients cope with distress related to symptom experience[6,7]. The best and longest lasting treatment results for IBS have been found in a combination of medical and psychological interventions[8].

COMORBIDITY

The overall burden of IBS is affected in part by high comorbidity of other medical and psychological disorders. Approximately 65% of patients with IBS have comorbid extra-intestinal symptoms and disorders[9] such as fibromyalgia[10], back pain[11], urogenital problems[12], sleep problems[13]. Additionally, 40%-60% of IBS patients (compared to 20% of the overall population) report comorbid psychiatric diagnoses such as anxiety disorders, depression, and Post Traumatic Stress Disorder[14,15]. Patients with IBS are also more likely to report low quality of life[16] and up to 38% of IBS patients in tertiary care settings have contemplated suicide as a result of their symptoms[17].

Given the high psychiatric comorbidity and increased stress-reactivity associated with IBS, several psychological interventions have been tailored to specifically target psychosocial skills deficits in IBS patients. Cognitive behavioral therapy (CBT) is the most commonly used and most widely researched psychological treatment for IBS to date. In this treatment, patients are taught to evaluate the relationships between thoughts, behaviors, and emotions and to cope with stressors by learning to modify maladaptive behaviors and to reframe unhelpful cognitions with the goal of improving mood and decreasing stress-reactivity. By doing so, patients can learn to decrease autonomic arousal, which may eventually decrease visceral hypersensitivity and reduce IBS symptoms[18,19]. Newer adaptations of the CBT model have also begun to incorporate data that supports the role of brain-gut dysregulation and symptom-specific processes (*i.e.*, symptom-specific anxiety) in the onset and maintenance of IBS[20,21]. These studies have supported the effectiveness of CBT in treating both physiological and psychological symptoms associated with IBS. For example, in a research study evaluating the effectiveness of 2 different types of CBT compared to Wait List Control, Lackner *et al*[21] demonstrated that 61%-72% of patients who received CBT treatment reported adequate relief of symptoms compared to 7.4% of Wait List Control patients.

BURDEN OF IBS

Due to currently insufficient medical interventions for IBS, the burden of living with IBS is quite high. As mentioned above, research has established that patients with IBS have high rates of psychopathology, low quality of life, and increased suicidal ideation. In addition, these patients miss more days of work, are less productive at work, and use many healthcare resources.

PSYCHOLOGICAL BURDEN

The primary goal of psychological interventions for IBS is to ease the overall burden of the illness. Decades of research using psychological parameters have provided a clear understanding of the psychological burden associated with IBS. As mentioned above, approximately 40%-60% of IBS patients have comorbid psychiatric diagnoses[14] and 38% contemplate suicide as a result of their symptoms[17]. Furthermore, these patients report lower quality of life than other patients with serious conditions such as end-stage renal disease or diabetes mellitus[16]. Research studies evaluating the psychological burden of IBS typically rely on validated self-report quality of life measures to evaluate the impact of glycemicindex symptoms of overall well-being[16]. These measures evaluate emotional and physical functioning together, without providing a clear or specific picture of the impact of IBS on daily activities.

WORK PRODUCTIVITY AND HEALTH-CARE UTILIZATION

A separate body of research has evaluated the burden of IBS on work-productivity and health care utilization[22,23]. This research reveals increased levels of both absenteeism and presenteeism in the workplace when compared to healthy controls. One study estimates that, assuming an IBS prevalence rate of 10%, an employer with 10000 employees could lose $7737600 per year in lost work-productivity due to IBS[22]. Furthermore, IBS accounts for 1.5-2.7 million physician visits a year, frequently resulting in unnecessary, expensive, and invasive diagnostics[24]. For example, 18%-33% of

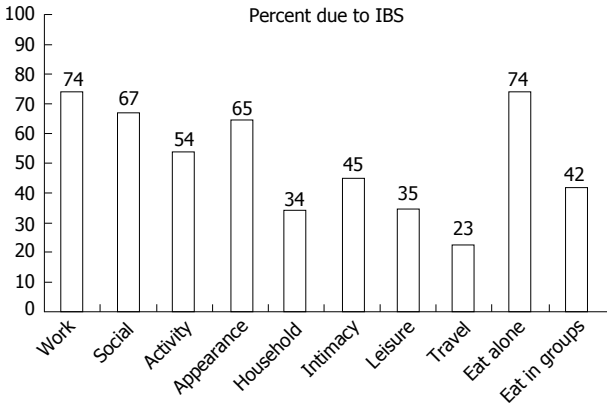

Figure 1 Percent of the sample (*n* = 35) that reported impairment in each area of daily living due to irritable bowel syndrome. IBS: Irritable bowel syndrome.

women with IBS have had a hysterectomy, compared to 12% to 17% of women without IBS who have had this same surgery[25,26].

DAILY FUNCTIONING

While many studies have evaluated the costly effects of IBS on psychological functioning, healthcare utilization, and work productivity, relatively few studies have focused on overall daily functioning in patients with IBS. Survey studies have shown that IBS patients report higher levels of difficulty in a broad range of daily activities when compared to healthy controls[27]; that IBS negatively affects both mental and physical functioning[16]; and that the reported effect of IBS on daily living is almost as high as that of the flu[28]. Activities that appear to be particularly impaired by IBS include: Work, intimacy, leisure activities, personal relationships, and eating habits[1,28,29]. However, these findings typically come from large survey studies and only one study to our knowledge has sought to quantify functional impairment among IBS patients[29]. The findings from that study suggested high levels of avoidance in a range of daily activities, particularly when symptoms were present.

The existing body of research on the impact of IBS on daily activities has only just begun to address the issue of the burden of IBS on daily functioning. Although most clinicians who work with IBS would agree that many of their patients modify or limit certain activities due to IBS symptoms (or fear of symptoms), this has not been adequately measured or quantified in this population.

FUNCTIONAL IMPAIRMENT IN IBS

Existing studies on functional impairment in IBS have offered, but not evaluated, two possible hypotheses for impaired daily functioning in IBS patients: (1) IBS symptoms may directly impact activities of daily living; and (2) emotional distress and/or maladaptive coping skills may primarily disrupt daily functioning. These hypotheses are consistent with current research in chronic pain suggesting that daily functioning is influenced by both emotional distress and actual physical pain symptoms[30]. To our knowledge, no research study has systematically evaluated the impact of both symptom severity and emotional distress on daily functioning in IBS patients.

A preliminary evaluation of functional impairment in a small sample (*n* = 35) of women with IBS suggests that patients avoid or are unable to participate in a wide range of activities, which they attribute to IBS symptoms (Figure 1). Interestingly, this preliminary data revealed that although most participants attributed functional impairment to IBS, symptom severity was not a significant predictor of functional impairment. In fact, symptom-specific anxiety and depression were the only significant predictors of impairment in a regression model that included symptom-specific anxiety, psychological distress, and symptom severity[31]. These findings are in-line with literature in pain and anxiety disorders suggesting that functional impairment may be independent of symptom severity. However, further research is required to evaluate the pathways that may lead to functional impairment.

CONCLUSION

Although the psychosocial and economic burden of IBS has been well documented, further research is necessary to evaluate the impact of IBS on daily activities. As mentioned above, existing measures of quality of life evaluate emotional and physical functioning together and do not provide a clear or specific understanding of the behavioral consequences of IBS (*e.g.*, avoiding social activities, avoiding work, avoiding travel, *etc.*). Existing research has alluded to behavioral avoidance or inability to participate in daily activities[27-30] but this concept has not yet been adequately or systematically characterized in IBS patients. Further research should evaluate and characterize functional impairment in IBS.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Structural brain lesions in inflammatory bowel disease

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Abstract

Central nervous system (CNS) complications or manifestations of inflammatory bowel disease deserve particular attention because symptomatic conditions can require early diagnosis and treatment, whereas unexplained manifestations might be linked with pathogenic mechanisms. This review focuses on both symptomatic and asymptomatic brain lesions detectable on imaging studies, as well as their frequency and potential mechanisms. A direct causal relationship between inflammatory bowel disease (IBD) and asymptomatic structural brain changes has not been demonstrated, but several possible explanations, including vasculitis, thromboembolism and malnutrition, have been proposed. IBD is associated with a tendency for thromboembolisms; therefore, cerebrovascular thromboembolism represents the most frequent and grave CNS complication. Vasculitis, demyelinating conditions and CNS infections are among the other CNS manifestations of the disease. Biological agents also represent a risk factor, particularly for demyelination. Identification of the nature and potential mechanisms of brain lesions detectable on imaging studies would shed further light on the disease process and could improve patient care through early diagnosis and treatment.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Structural lesions; Magnetic resonance imaging; Brain lesions

Core tip: Central nervous system complications or manifestations of inflammatory bowel disease deserve particular attention because symptomatic conditions can require early diagnosis and treatment, whereas unexplained manifestations might be linked to pathogenic mechanisms. This review focuses on both symptomatic and asymptomatic brain lesions detectable on imaging studies, as well as their frequency and potential mechanisms. A direct causal relationship between inflammatory bowel disease and asymptomatic structural brain changes has not been demonstrated, but several possible explanations, including vasculitis, thromboembolism and malnutrition, have been proposed. Identification of the nature and potential mechanisms of

brain lesions on imaging studies would improve patient care through early diagnosis and treatment.

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INTRODUCTION

Inflammatory bowel diseases (IBDs), namely ulcerative colitis (UC) and Crohn's disease (CD), are chronic, debilitating conditions with their onset at relatively young ages. CD is a transmural disease of gastrointestinal mucosa, and it has the potential to affect the entire gastrointestinal tract; in contrast, UC is not a transmural disease, and it affects the colon[1,2]. Both have relapsing and remitting courses. It is estimated that the total (UC plus CD) prevalence of IBD is approximately 0.4% in the Western populations[3].

Because IBD can involve body parts other than the gastrointestinal tract, it can be regarded as a systemic disease. Involvement of the skin, eyes, joints, liver, biliary tract, kidneys, and bone, as well as hematological, and neurological involvement, can occur, preceding, accompanying or following gastrointestinal symptoms. These extraintestinal manifestations are more common in CD patients, although a substantial number of IBD patients can develop these conditions[4-6].

Neurological manifestations are relatively rare but are of clinical importance, particularly in terms of the need for timely diagnosis and management. Several mechanisms, including thromboembolisms, immunologic abnormalities, drug side effects, malabsorption, and infections, have been suggested as pathogeneses[7]. Most central neurological manifestations of IBD can be detected by brain imaging studies because they cause structural alterations of neural structures to some extent. However, lesions of unknown clinical relevance have also been detected in these patients, with significantly higher prevalence than in the normal population.

This review focuses in particular on structural brain lesions with positive findings on imaging studies, including symptomatic conditions and asymptomatic situations with brain lesions as well (Table 1).

ASYMPTOMATIC STRUCTURAL CHANGES ON MAGNETIC RESONANCE IMAGING

To date, a small number of studies have examined the presence of white matter lesions and other structural alterations on imaging studies in patients with IBD. These lesions were asymptomatic with potential associations with IBD, and whether these structural changes represent a unique extraintestinal manifestation of the disease remains unclear. Figure 1 depicts white matter and gray matter (GM) on an magnetic resonance imaging (MRI) scan.

Initial reports examining the associations between IBD and asymptomatic brain lesions were conflicting[8,9]. In a study by Geissler *et al*[8], 72 patients with IBD (48 cases of CD and 24 of UC) and 50 healthy age-matched controls underwent magnetic resonance imaging with gadolinium-enhanced studies. In that series, hyperintense focal white-matter lesions of 2-8 mm in diameter were found in 42% and 46% of patients with CD and UC, respectively, whereas such lesions were only present in 16% of healthy controls, resulting in relative risks of 2.6 for CD (95%CI: 1.3-5.3) and 2.9 for UC (95%CI: 1.3-6.2). A longer duration of disease and older age were associated with an increased tendency for the lesions, and none of the patients had neurological symptoms. In contrast to the study by Geissler *et al*[8], Hart *et al*[9] did not find a significantly increased frequency of asymptomatic brain white matter lesions on the MRIs of IBD patients (n = 40), compared to a control group consisting of 40 age- and sex-matched patients admitted for tension-type headache (12.5% *vs* 5%, P = 0.43). Although the relatively small sample size of the latter study might have prevented the differences from attaining statistical significance, the authors emphasized that such asymptomatic lesions had previously been reported consistently in healthy subjects[10], and they expressed concerns about the clinical relevance of these findings for patients with IBD. However, it should be emphasized that both reports dated from two decades ago. In contrast, a recent study with a relatively small sample size also could not find an increased rate of white matter lesions among patients with IBD, compared to healthy controls[11]. In that study, the frequencies of white matter lesions and other brainstem parenchymal lesions were similar, but among the subjects with white matter lesions, the number of lesions was significantly higher in IBD patients.

Two recent studies compared the frequency of white matter lesions between IBD patients and normal subjects using advanced magnetic resonance imaging techniques and equipment[12,13]. Chen *et al*[12] found a very high prevalence of hyperintense white matter lesions in patients with CD, compared to age-matched controls (75% *vs* 34%, $P < 0.001$). Their study had a relatively large sample size (54 Crohn's patients and 100 age-matched controls). Similarly, Zikou *et al*[13] found a significantly increased frequency of white matter lesions among patients with IBD (Crohn's and UC), compared to controls (66% *vs* 45%, $P < 0.05$). Both studies used fluid-attenuated inversion recovery images to evaluate white matter hyperintensities.

The development of advanced MRI techniques has allowed for better examination of brain structures, including estimation of volume differences and the evaluation of microstructural integrity. Voxel-based morphometry is a technique used to compare the regional

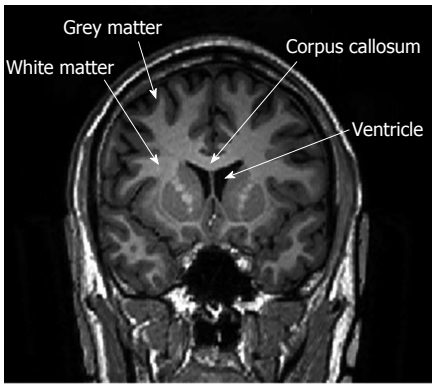

Figure 1 T1-weighted magnetic resonance image of a coronal section through the brain. Gray matter and white matter are indicated, as well as the ventricles.

brain volumes of subjects, and it uses MRI data. Several studies using this technique have found a decreased GM volume among IBD patients, compared to controls. Agostini *et al*[14], in a study of CD patients, found decreased GM volume in parts of the frontal gyrus and in the anterior midcingulate cortex. In addition, negative correlations between disease duration and GM volume in several brain regions were found in the former study. In contrast, a recent study by the same lead author did not identify any decrease in GM volume in UC patients, compared to controls[15]. Zikou *et al*[13], however, found more diffuse GM volume decreases in a sample of IBD patients, consisting of subjects with CD and UC, involving the right and left fusiform gyri, the right and left temporal inferior gyri, the right precentral gyrus, the right superior motor area, the right middle frontal gyrus and the left superior parietal gyrus.

Diffusion tensor imaging (DTI) is an MRI technique that measures molecular diffusion. It is valuable for the identification of axonal injury and can be useful in differentiating between primary and Wallerian degeneration[16]. To date, only a single study has examined the brains of IBD patients using DTI techniques and compared them with age-matched healthy controls[13]. That study found decreased white matter axial diffusivity in the right corticospinal tract and the right superior longitudinal fasciculus in IBD patients (Crohn's and UC) compared to controls, indicating a possible degree of change in neural structures[17-21].

Several possible explanations have been proposed for the increased prevalence of white matter lesions, the decreased GM volume, and the decreased diffusivity in major tracks among patients with IBD. White matter lesions on brain MRI examinations have been associated with many conditions, including migraine headaches, hypertension, diabetes, celiac disease, and cerebrovascular disease, as well as being found in healthy subjects[10,22-26]. The increased frequency of white matter hyperintensities in IBD patients might be due to central nervous system (CNS) vasculitis, likely secondary to coagulation and vessel obstruction[13]. IBD is known to be associated with thromboembolism and hypercoagulability[27-29]. Another possible explanation is malabsorption because such lesions were previously described in celiac disease[26].

Table 1 Structural brain lesions

Asymptomatic structural changes
Symptomatic structural changes
Cerebrovascular lesions
Demyelinating conditions
Cerebral vasculitis
CNS infections

CNS: Central nervous system.

One explanation for decreased GM volume might be excito-toxicity due to chronic pain, which can result in neural atrophy or loss[14,30]. Structural changes of varying types have been observed in pain-related brain regions in other chronic pain syndromes[31-36], with GM decreases in the frontal and cingulate cortices being the most common anomalies[30]. Another possible mechanism might be increased inflammatory cytokines, resulting in astrocyte and oligodendrocyte apoptosis, decreased neurogenesis, and increased oxidative stress, thus leading to GM volume loss[37,38]. Similarly, cerebral small vessel vasculitis and the neurotoxic effects of cytokines could be responsible for the decreased axial diffusivity in major tracts[13].

Although a direct causal relationship between IBD and asymptomatic structural brain changes cannot be established, the available findings and their implications deserve further investigation, particularly in the context of the brain functional changes observed in patients with IBD[39].

CEREBROVASCULAR LESIONS

Patients with IBD have an increased tendency for thrombotic events so that IBD could be considered a prothrombotic condition that increases the risk of cerebral arterial and venous thrombosis[40-44]. A recent meta-analysis found a modestly increased risk of cerebrovascular accidents among patients with IBD (OR = 1.18)[45]. The increased risk was more prominent among female patients and young patients. Venous thrombosis has also been seen in these patients with remarkable frequency[44,46-49]. Cerebrovascular events have been documented in up to 4% of IBD patients[50]. These cases are of particular clinical importance when the consequences of these conditions and the young age of the patient population are considered.

A number of pathological conditions have been linked to IBD in attempts to propose a mechanism for the increased tendency for thromboembolic events, including abnormalities of platelets and coagulation factors, genetic mutations, vitamin B6 deficiency due to hypercatabolism and malabsorption, antiphospholipid antibodies, hyperhomocystinemia, dehydration, and immobilization[4,7,40,49,51-58]. However, to date, the exact mechanism has not been fully understood.

Cerebral arterial thromboembolism can present with headache, paresis, seizures or dysphagia, and it can result in high mortality and morbidity[51]. Large infarcts involving both the anterior and posterior circulation and lacunar infarcts have been reported in IBD[59-63]. In addition, UC has been associated with thrombotic thrombocytopenic purpura and small and large cerebral artery thrombosis risks[64-66]. Infarcts associated with IBD can be identified on computerized tomography and magnetic resonance imaging.

Cerebral venous thrombosis and sinus thrombosis seem to be more frequent in patients with UC than in CD patients[67]. Most often, the superior sagittal sinus and lateral sinuses are involved; however, thrombosis of the cortical venous sinuses has also been reported[68]. Young and male patients seem to be at greater risk[50,69,70]. The most common presenting symptom is headache, usually followed by neurological impairment, and cerebral infarction can develop due to extension of thrombus[71]. A combination of magnetic resonance imaging and magnetic resonance venography could identify venous occlusion[71]. The radiological characteristics, as well as the clinical course and prognosis of IBD-related cerebral venous thrombosis, seem to be similar to those in cases not related to IBD[44,72,73].

DEMYELINATING CONDITIONS

Demyelinating conditions have been reported in the setting of IBD, in association or not with biological treatments.

Multiple sclerosis (MS) or MS-like conditions have long been reported in patients with IBD[74-77]. Such a relationship was first reported by Rang *et al*[74]. A retrospective study found an increased incidence of demyelinating disease among patients with IBD, particularly in UC[77]. Nevertheless, MS has been reported in both UC and CD[78,79]. The findings of a recent meta-analysis supported a relationship between MS and IBD[80]. Both the development of an MS-like syndrome in the setting of IBD and the development of IBD in MS patients have been reported[81-83]. Because the diagnostic criteria for MS have evolved over time, some lesions found in previous studies might not actually be MS; rather, they could represent an MS-like syndrome, which might potentially be linked to IBD. The mechanism of these relationships has not been fully explained, but a role for impairments in functional T-cell subsets has been proposed[4,57].

Anti-tumor necrosis factor (TNF)-alpha drugs and anti-alpha4 integrin drugs (such as natalizumab), which are biological agents used in the treatment of IBD, can have adverse neurological effects[84,85]. Progressive multifocal leukoencephalopathy is the gravest complication, particularly when associated with natalizumab therapy, although few cases have been reported in association with TNF-alpha drugs[85]. Reactivation of John Cunningham virus (JCV) is responsible for the development of PML, and it is associated with visual defects, mental impairment, confusion and personality changes, followed by motor weakness[57]. MRI is helpful in the diagnosis, showing white matter lesions with typical T1 and T2 signals[86]. Diagnosis can be confirmed by polymerase chain reaction for JCV DNA. Despite treatment, PML has a high mortality rate of 60%[87]. In addition, development/exacerbation of MS or demyelination has been reported in association with anti-TNF-alpha therapy[88-90].

CEREBRAL VASCULITIS

Cerebral vasculitis has been reported in patients with UC in a number of studies[91-96]. In addition, a case of cerebral vasculitis was reported in association with CD[97]. Mostly immune-mediated mechanisms have been proposed for the development of vasculitis in UC. Magnetic resonance imaging is abnormal and shows hyperintense lesions[95,96], and magnetic resonance angiography can aid in diagnosis[97]. CNS vasculitis has also been reported in association with anti-TNF therapy[57]. The major symptoms of cerebral vasculitis are stroke, headache and encephalopathy. Other symptoms include seizures, cranial nerve palsies or myelopathies[98]. Cerebral vasculitis mimicking migraine with aura was reported in a case of CD, and the authors stated that migraine with aura can be the only finding in cerebral vasculitis[99]. Cerebral vasculitis resulting in stroke has been rarely reported in UC[61]. Cerebral vasculitis presented with right paresis and unbalanced gait in a 35-year-old woman with UC[93]. Another UC case was complicated by convulsions and was diagnosed as cerebral vasculitis on magnetic resonance imaging[96].

CNS INFECTIONS

Anti-TNF agents can suppress the immune system to such an extent that opportunistic infections develop, including of the CNS, in IBD patients. These patients present with meningeal signs, seizures, symptoms resembling stroke, and encephalopathy[100]; abnormal MRI findings and/or mass lesions are found on imaging studies. Among these opportunistic infections are fungal infections, cerebral tuberculosis, Epstein-Barr virus infection, nocardiosis, toxoplasmosis, herpes simplex virus infection, meningococcal infection, *Campylobacter fetus* infections, and listeria infections[57]. In a severe case of CD with ileocecal involvement, opportunistic meningitis with varicella zoster was reported after adalimumab and prednisone treatment[101]. In a patient with CD, meningococcal meningoencephalitis was reported after certolizumab pegol treatment[102].

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REVIEW

Elusive liver factor that causes pancreatic α cell hyperplasia: A review of literature

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Abstract

Tumors and cancers of the gastrointestinal tract and pancreas are commonly derived from precursor lesions so that understanding the physiological, cellular, and molecular mechanisms underlying the pathogenesis of precursor lesions is critical for the prevention and treatment of those neoplasms. Pancreatic neuroendocrine tumors (PNETs) can also be derived from precursor lesions. Pancreatic α cell hyperplasia (ACH), a specific and overwhelming increase in the number of α cells, is a precursor lesion leading to PNET pathogenesis. One of the 3 subtypes of ACH, reactive ACH is caused by glucagon signaling disruption and invariably evolves into PNETs. In this article, the existing work on the mechanisms underlying reactive ACH pathogenesis is reviewed. It is clear that the liver secretes a humoral factor regulating α cell numbers but the identity of the liver factor remains elusive. Potential approaches to identify the liver factor are discussed.

Key words: Pancreatic α cell hyperplasia; Humoral factor; Pancreatic neuroendocrine tumors; Digestive system hormone; Liver

Core tip: Tumors and cancers of the gastrointestinal tract and pancreas are commonly derived from precursor

lesions. One of the precursor lesions, reactive pancreatic α cell hyperplasia is caused by glucagon signaling disruption and invariably evolves into pancreatic neuroendocrine tumors. In this article, the existing work on the mechanisms underlying the novel precursor lesion is reviewed. It is clear that the liver secretes a humoral factor regulating pancreatic α cell numbers but the identity of the liver factor remains elusive. Potential approaches to identify the liver factor are discussed.

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INTRODUCTION

Tumors and cancers of the gastrointestinal tract and pancreas are commonly derived from precursor lesions[1-3]. For example, colon cancer is derived from polypoid or non-polypoid pre-neoplastic lesions in the colon, and pancreatic ductal carcinoma from pancreatic intraepithelial neoplasia. Neuroendocrine tumors in the gastrointestinal tract and pancreas (GEP-NETs) are relatively rare and indolent tumors with variable biological behaviors[4-6]. GEP-NETs can also be derived from precursor lesions[7-9]. In atrophic gastritis, hypergastrinemia drives entero-chromaffin-like cell hyperplasia, which in turn can give rise to gastric carcinoids[7,10]. In ulcerative colitis, microscopic neuroendocrine tumors can arise after long disease duration, probably in response to inflammation[7,11]. Recently, precursor lesions giving rise to pancreatic neuroendocrine tumors (PNETs) have drawn much attention and become more understood. It is well known now that diffuse precursor lesions including endocrine cell hyperplasia, dysplasia, and microadenomas are present in the pancreata of patients with familial tumor syndromes such as multiple endocrine neoplasia syndrome type 1 (MEN1) and von Hippel-Lindau disease, and of animal models of PNETs[12-16]. In the pancreata of patients with MEN1 and mice with heterozygous MEN1 inactivation, the hyperplastic endocrine cells are polyclonal and multi-hormonal and contain the normal menin allele, while microadenomas have to first lose the normal menin allele[17,18]. In contrast, uni-hormonal pancreatic endocrine cell hyperplasia such as pancreatic α cell hyperplasia (ACH) and pancreatic polypeptide cell hyperplasia has only been recognized in the last several years[8,9,19]. Although pancreatic polypeptide cell hyperplasia may be a physiological variation of normal pancreatic polypeptide cell distribution, ACH is clearly a pathologic precursor lesion leading to PNET pathogenesis[19].

In this article, we will summarize how the discovery of a novel hereditary tumor syndrome, Mahvash disease, has stimulated interest in the pathogenesis of ACH, and discuss the possible identify of an elusive liver factor that may cause the ACH.

The data we review are based on work in our own laboratories and PubMed and major endocrine conferences search using key words pancreatic α cell hyperplasia, glucagon receptor mutation, glucagon receptor antagonism, and hyperglucagonemia.

PANCREATIC ACH

ACH is defined as an overwhelming and specific increase of pancreatic α cell numbers[8,19]. Based on etiology and glucagon levels, 3 types of ACH are observed. Reactive ACH is caused in humans by inactivating glucagon receptor mutations and is associated with marked hyper-glucagonemia. Because the glucagon receptor is inactive, the severe hyperglucagonemia in reactive ACH does not result in glucagonoma syndrome. Non-functional ACH has an unknown cause and is associated with normal glucagon levels. Functional ACH also has an unknown cause but is associated with hyperglucagonemia that results in glucagonoma syndrome.

Reactive ACH is most extensively studied due to the novel Mahvash disease and the existence of multiple animal models. We first described the Mahvash disease which is hyperglucagonemia, ACH, and PNETs but without glucagonoma syndrome, caused by an inactivating glucagon receptor mutation[20,21]. Later, we and others have confirmed the Mahvash disease (Tang L and Yu R, unpublished results)[19,22]. Currently, 8 inactivating glucagon receptor mutations are known.

We further established that the glucagon receptor knockout ($Gcgr^{-/-}$) mice are a murine model of Mahvash disease[23-25]. The $Gcgr^{-/-}$ mice exhibit ACH throughout their lifespan. Dysplastic islets consisted of mostly α cells are evident from 5-7 mo on and glucagonomas are detected from 10-12 mo to death. Hyperplasia is also observed in the exocrine compartment but dysplasia, carcinoma *in situ*, or frank exocrine carcinoma is not found. Large PNETs contribute at least partially to the premature demise of the $Gcgr^{-/-}$ mice. Three other murine models also mimic the Mahvash disease in some aspects. The prohormone convertase 2 knockout ($PC^{-/-}$) mice cannot make mature glucagon; they exhibit ACH and eventually develop PNETs[26,27]. The preproglucagon knockout ($Gcg^{-/-}$) mice cannot make any proglucagon-derived peptide hormones, including mature glucagon; they also exhibit ACH and eventually develop PNETs[28,29]. The liver-specific Gsα knockout mice cannot transduce the glucagon signaling in hepatocytes; they exhibit hyperglucagonemia and ACH, and eventually develop PNETs as well[30,31].

Thus both in humans and in mice, reactive ACH ensues whenever glucagon signaling is disrupted and evolves into PNETs eventually. Reactive ACH thus is clearly a precursor lesion leading to PNET pathogenesis.

PATHOGENESIS OF REACTIVE ACH

The pathogenesis of reactive ACH in $Gcgr^{-/-}$ mice is

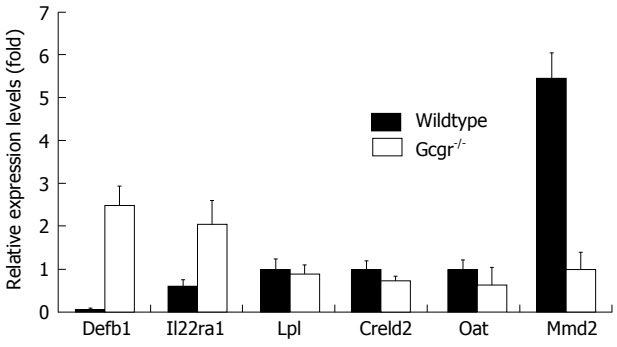

Figure 1 Realtime polymerase chain reaction of several genes differentially expressed in the Gcgr$^{-/-}$ mice. See text for details.

studied in detail. As increased pancreatic endocrine cell numbers can be due to proliferation, neogenesis, or reduced apoptosis, they are each examined at 6-7 mo[23]. α cell proliferation measured by proliferating cell nuclear antigen or Ki-67 labeling is very low and not significantly different in WT and Gcgr$^{-/-}$ mice. α cell neogenesis measured by counting singlet and doublet α cells and exocrine ducts harboring glucagon-positive cells is much higher in Gcgr$^{-/-}$ than in WT mice. α cell apoptosis measured by TUNEL labeling is very low in both Gcgr$^{-/-}$ and WT mice and not significantly different. While upregulated α cell neogenesis is also seen in the PC$^{-/-}$ mice throughout their lifespan, higher α cell proliferation is found at 3 mo[27].

The hyperplastic α cells in the Gcgr$^{-/-}$ mice exhibit abnormal differentiation. A few of these cells are positively labeled with both glucagon and insulin, and some express pancreatic and duodenal homeobox 1, a β cell marker[23]. Most α cells express embryonic α cell markers such as GLUT2[32]. Abnormal α cell differentiation is also seen in humans with mutated Gcgr and in the PC$^{-/-}$ mice as they both express glucagon-like peptide 1, which is normally not expressed in the α cells[21,27].

REACTIVE ACH AND THE LIVER

As reactive ACH universally occurs after glucagon signaling inhibition (see above), it is logical to hypothesize that glucagon signaling negatively feeds back on α cell number regulation and loss of the negative feedback causes the ACH[33]. A number of lines of evidence point to the liver as the organ which sends inhibitory signals to the α cells during normal glucagon signaling and a stimulatory signal to them when glucagon signaling is disrupted. First, liver is the natural target organ of glucagon signaling. Second, liver-specific Gsα deletion in mice recapitulates ACH pathogenesis[30]. Third, liver-specific glucagon receptor deletion in mice results in a phenotype very similar to that of mice with global glucagon receptor deletion[34]. Fourth, glucagon receptor re-expression in the liver of Gcgr$^{-/-}$ mice reduces glucagon levels by almost 99%[35]. Therefore, if the liver does not respond to glucagon but all other organs do, reactive ACH ensues; conversely, if the liver does respond to glucagon but all other organs do not, reactive ACH likely reverses. In other words, the liver is likely necessary and sufficient to be the organ regulating the number of α cells in response to glucagon signaling.

THE ELUSIVE LIVER FACTOR

The liver communicates with the pancreas *via* neuronal and humoral signals. It has been shown that the liver can regulate insulin secretion and pancreatic β cell proliferation through neuronal signals[36-38]. In a similar manner, the liver may regulate glucagon secretion and pancreatic α cell proliferation through neuronal connection, but there has not been any direct experimental evidence supporting or disputing that. In contrast, there is strong evidence that the liver regulates glucagon secretion and pancreatic α cell proliferation through a humoral factor as shown by islet transplantation experiments[34]. Transplanted wildtype islets in Gcgr$^{-/-}$ recipient mice exhibit higher α/β cell ratio and increased α cell proliferation, compared with those in wildtype recipient animals. Conversely, transplanted Gcgr$^{-/-}$ islets in wildtype recipient mice exhibited reduced α-cell proliferation compared with those in Gcgr$^{-/-}$ recipient animals.

The nature and identity of the liver factor that causes reactive ACH have been sought after. As the liver gene expression must be different between the wildtype and the Gcgr$^{-/-}$ mice, systems approaches such as DNA microarray studies are done to efficiently provide systemic and novel insights into the nature of the liver factor. We compared gene expression profile of 4 WT and 4 Gcgr$^{-/-}$ mouse livers at 2.5 mo (2 females and 2 males in each group) by Affymetrix GeneChip Mouse Gene 1.0 ST Array. The microarray data were analyzed using Genespring 11 (Tables 1 and 2). A total of 125 genes were significantly differentially expressed (> 2 fold change and $P < 0.05$). Since ACH occurs regardless of sex, we eliminated 47 genes with differential expression only limited in one sex, leaving 35 genes upregulated and 43 genes downregulated in both female and male Gcgr$^{-/-}$ mouse liver. The differential expression of some of the 78 genes was validated by realtime polymerase chain reaction (Figure 1). We reason that potential candidate genes should encode secretory proteins. Of the genes overexpressed in Gcgr$^{-/-}$ liver, Igfbp1, Defb1, Serpina7, Inhba, Cxcl13, Il1b, and Cxcl9 are secretory proteins and may stimulate α-cell differentiation and proliferation. Defb1 is particularly interesting as it is very significantly overexpressed in the Gcgr$^{-/-}$ liver (Table 1). Defensins are a group of cysteine-rich antimicrobial peptides that function to help defend against microbial infections[39]. They are mostly secreted by leukocytes and epithelial cells and their anti-microbial mechanisms are multiple. There are a few families of defensins according to their structures in mice and humans. Originally, defensin β1 (DB1, encoded by Defb1) is found to be expressed in the lung and urogenital epithelials cells[40-42]. Later, DB1 is also found

Table 1 Genes significantly overexpressed in the Gcgr$^{-/-}$ mouse liver

Gene symbol	mRNA description	GO biological process term	Fold increase
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21), transcript variant 1	Response to DNA damage stimulus/cell cycle/cell cycle arrest/negative regulation of cell proliferation	5.6
Igfbp1	Insulin-like growth factor binding protein 1	Regulation of cell growth	5.5
Defb1	Defensin beta 1	Defense response/response to bacterium/defense response to bacterium/innate immune response	5.2
Gpr64	G protein-coupled receptor 64, transcript variant 1	Signal transduction/cell surface receptor linked signaling pathway/G-protein coupled receptor protein signaling pathway/neuropeptide signaling pathway	5.1
Serpina7	Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	Post-embryonic development/response to vitamin A/response to drug	4.9
Cpt1b	Carnitine palmitoyltransferase 1b, muscle, nuclear gene encoding mitochondrial protein	Lipid metabolic process/fatty acid metabolic process/transport/long-chain fatty acid transport/long-chain fatty acid transport	4.6
Chac1	ChaC, cation transport regulator-like 1 (*E. coli*)	Apoptosis/response to unfolded protein/biological_process	4.3
Npas2	Neuronal PAS domain protein 2	Transcription/regulation of transcription, DNA-dependent/signal transduction/circadian sleep/wake cycle/regulation of transcription/locomotor rhythm/positive regulation of transcription from RNA polymerase Ⅱ promoter/rhythmic process	3.9
Slc34a2	Solute carrier family 34 (sodium phosphate), member 2	In utero embryonic development/transport/ion transport/sodium ion transport/phosphate transport/phosphate transport/phosphate transport	3.6
Fabp5	Fatty acid binding protein 5, epidermal	Glucose metabolic process/lipid metabolic process/phosphatidylcholine biosynthetic process/transport/glucose transport	3.5
BC023105	cDNA sequence BC023105	Unknown	3.1
Cav1	Caveolin 1, caveolae protein	MAPKKK cascade/inactivation of MAPK activity/vasculogenesis/response to hypoxia/negative regulation of endothelial cell proliferation/negative regulation of cytokine-mediated signaling pathway/triglyceride metabolic process/calcium ion transport/cellular calcium ion homeostasis/cellular calcium ion homeostasis/endocytosis/regulation of smooth muscle contraction	2.8
Lpl	Lipoprotein lipase	Lipid metabolic process/positive regulation of macrophage derived foam cell differentiation/lipid catabolic process/triglyceride biosynthetic process/triglyceride catabolic process	2.7
Il22ra1	Interleukin 22 receptor, alpha 1	Blood coagulation	2.6
Acaca	Acetyl-Coenzyme A carboxylase alpha	Tissue homeostasis/acetyl-CoA metabolic process/lipid metabolic process/fatty acid biosynthetic process/metabolic process/lipid biosynthetic process/response to organic cyclic substance/multicellular organismal protein metabolic process	2.6
Inhba	Inhibin beta-A	Mesoderm formation/hemopoietic progenitor cell differentiation/growth/positive regulation of transcription from RNA polymerase Ⅱ promoter/mesodermal cell differentiation/negative regulation of hair follicle development	2.6
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	Activation of MAPKK activity/negative regulation of protein kinase activity/apoptosis/multicellular organismal development/cell differentiation/regulation of cell cycle	2.4
Tgtp1	T-cell specific GTPase 1	Immune response/response to virus	2.4
Rassf4	Ras association (RalGDS/AF-6) domain family member 4	Cell cycle/signal transduction	2.4
Cxcl13	Chemokine (C-X-C motif) ligand 13	Chemotaxis/inflammatory response/immune response/lymph node development	2.4
Il1b	Interleukin 1 beta	Angiogenesis/fever/inflammatory response/immune response/elevation of cytosolic calcium ion concentration/aging	2.4
Tgtp1	T-cell specific GTPase 1	Immune response/response to virus	2.3
Asns	Asparagine synthetase	Asparagine biosynthetic process/glutamine metabolic process/metabolic process/cellular amino acid biosynthetic process	2.3
Socs2	Suppressor of cytokine signaling 2, transcript variant 1	Lactation/regulation of growth/regulation of multicellular organism growth/negative regulation of multicellular organism growth/negative regulation of multicellular organism growth/positive regulation of neuron differentiation/negative regulation of JAK-STAT cascade/mammary gland alveolus development	2.3
Meig1	Meiosis expressed gene 1	Meiosis	2.3
Cxcl9	Chemokine (C-X-C motif) ligand 9	Inflammatory response/immune response	2.2
Vtcn1	V-set domain containing T cell activation inhibitor 1	Negative regulation of T cell activation	2.2
H2-Ab1	Histocompatibility 2, class Ⅱ antigen A, beta 1	Antigen processing and presentation of peptide or polysaccharide antigen *via* MHC class Ⅱ/immune response/antigen processing and presentation/antigen processing and presentation of exogenous peptide antigen *via* MHC class Ⅱ	2.2

Spon2	Spondin 2, extracellular matrix protein	Cell adhesion/innate immune response	2.2
Rgs16	Regulator of G-protein signaling 16	G-protein coupled receptor protein signaling pathway/negative regulation of signal transduction	2.1
Il2rg	Interleukin 2 receptor, gamma chain	Regulation of gene expression/positive regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation/positive regulation of T cell differentiation in the thymus/positive regulation of B cell differentiation	2.1
Wdr67	WD repeat domain 67, transcript variant 1	Regulation of Rab GTPase activity	2.1
Prss8	Protease, serine, 8 (prostasin)	Hair follicle development/proteolysis	2.1
Bach2	BTB and CNC homology 2	Transcription/regulation of transcription, DNA-dependent/regulation of transcription	2.0
Serpina12	Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	Unknown	2.0

E. coli: *Escherichia coli*.

in the liver, especially in the biliary epithelial cells under obstructive jaundice[40,43,44]. DB1 released in circulation may result in ACH. Serpina7 encodes thyroxine-binding globulin (TBG) which binds thyroxine and increases total thyroxine levels[45]. As the index patient with Mahvash disease has normal thyroid functions, suggesting normal TBG levels, it is unlikely that TBG is the liver factor that causes ACH. For Cxcl13, Il1b, and Cxcl9, please see below. Literature review does not give us any clues on which underexpressed genes in Gcgr$^{-/-}$ mouse liver might encode a secretory protein that acts as inhibitor of α-cell differentiation and proliferation.

Pathway analysis suggested that WT and Gcgr$^{-/-}$ liver exhibit different metabolic profiles. As expected, genes involved in gluconeogenesis, glycogen synthesis, and glycogenolysis were downregulated in the Gcgr$^{-/-}$ liver, compared with those in WT (Table 3). Interestingly, genes involved in inflammation and cell proliferation were upregulated in the Gcgr$^{-/-}$ liver. The protein products of genes regulating cell proliferation unlikely diffuse out of the liver thus are improbable signals for regulating α cell mass. In contrast, the protein products of genes regulating inflammation are mostly cytokines which are secreted into the circulation and can reach the α cells, such as Cxcl13, Il1b, and Cxcl9. Interestingly, interleukin-6 (IL6), a cytokine secreted by T cells and macrophages (but not by the liver), upregulates α-cell mass but circulating IL6 levels are normal in the Gcgr$^{-/-}$ mice[34,46]. Alternatively, multiple liver-elaborated cytokines may act synergistically to cause ACH.

The liver may indirectly regulate α cell differentiation and proliferation by metabolic signals. Not surprisingly, the metabolic profile of Gcgr$^{-/-}$ mice and wildtype counterparts are vastly different, as shown by polyomic metabolic profiling[47]. Similar to our results, genes involved in gluconeogenesis and amino acid catabolism are downregulated. Furthermore, genes involved in fatty acid oxidation processes are also downregulated and genes involved in glycolysis, fatty acid synthesis, and cholesterol synthesis are upregulated. More pertinent to the potential mechanisms for ACH pathogenesis are the dramatic changes in the levels of metabolites[47]. As reported before[21,23], glucose levels are decreased by 1.4-fold. Consistent with decreased gluconeogenesis in the Gcgr$^{-/-}$ mice, amino acids and amino acid derivatives levels are significantly elevated. The most upregulated amino acids are threonine (9.6-fold), serine (8.7-fold), and asparagine (8.1-fold). Amino acid derivatives levels are also higher in the Gcgr$^{-/-}$ mice, the highest being 2-aminodipic acid and ornithine (both 5.4-fold). Levels of certain nucleotides and their derivatives are elevated, *e.g.*, pyridoxine levels are 3.6-fold elevated. Levels of some vitamins are different; those of dihydrofolic acid are 5.3-fold elevated. Glycerol and glycerol derivatives levels are about 2-fold lower. Intriguingly, the levels of cholic acid and glycocholic acid, two bile acids, are markedly and unexpectedly elevated (244- and 154-fold, respectively). There have been only a few studies addressing glucagon signaling and bile acids. In the rats, glucagon increases cholic acid levels[48]; in cultured cells, one bile acid, chenodeoxycholic acid, desensitizes the glucagon receptor[49]. Bile acids, however, are recognized recently as metabolic regulators[50]. Wildtype mice fed with cholic acid exhibit markedly elevated bile acid levels but their pancreas weight and glucagon levels are not changed[34]. Interestingly, α cell mass is somewhat increased (approximately 80%) by cholic acid feeding. Thus a metabolic signal that causes ACH has not been identified yet.

FUTURE DIRECTIONS

The elusive, yet-to-be identified liver factor that causes ACH fulfills the definition of a novel digestive system hormone (Figure 2). The liver factor is produced by the liver and released into the circulation; it then acts remotely on the pancreas to result in ACH. The liver factor could be more than one molecule but we use singular form here for conciseness. To identify this liver factor, the process of discovering leptin may offer some insights. When the first obese mouse models were described, it was not clear why they are obese. A circulating factor was hypothesized[51]. In the obese mouse models, the factor may either stimulate appetite and be overproduced or inhibit appetite and be under-produced. The circulating factor hypothesis was tested by parabiosis which joins the circulation of two mice of various lean and obese phenotypes. Eventually it was found that the *ob/ob* obese mice lack an inhibitor of appetite (leptin) and the *db/db* obese mice lack the

Table 2 Genes significantly underexpressed in the Gcgr$^{-/-}$ mouse liver

Gene symbol	mRNA description	GO biological process term	Fold decrease
Mmd2	Monocyte to macrophage differentiation-associated 2	Cytolysis	9.7
Nnmt	Nicotinamide N-methyltransferase	Unknown	6.2
Gcgr	Glucagon receptor	Exocytosis/signal transduction/cell surface receptor linked signaling pathway/G-protein coupled receptor protein signaling pathway/G-protein signaling, coupled to cAMP nucleotide second messenger/activation of adenylate cyclase activity by G-protein signaling pathway	5.3
Mfsd2a	Major facilitator superfamily domain containing 2A	Transport/transmembrane transport	4.2
Oat	Ornithine aminotransferase, nuclear gene encoding mitochondrial protein	Unknown	4.1
Slc10a2	Solute carrier family 10, member 2	Transport/ion transport/sodium ion transport/organic anion transport/bile acid and bile salt transport	3.9
A1bg	Alpha-1-B glycoprotein	Unknown	3.5
Gm129	Gene model 129 (NCBI)	Unknown	3.3
Sds	Serine dehydratase	Gluconeogenesis/cellular amino acid metabolic process/metabolic process	3.1
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	Gluconeogenesis/gluconeogenesis/oxaloacetate metabolic process/lipid metabolic process/glycerol biosynthetic process from pyruvate	3.0
Lrtm1	Leucine-rich repeats and transmembrane domains 1	Unknown	3.0
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2, transcript variant 1	Vasculogenesis/protein amino acid phosphorylation/transmembrane receptor protein tyrosine kinase signaling pathway/multicellular organismal development/nervous system development/feeding behavior/glutamate secretion/regulation of metabolic process/cell differentiation/brain-derived neurotrophic factor receptor signaling pathway/mechanoreceptor differentiation	3.0
Gls2	Glutaminase 2 (liver, mitochondrial), nuclear gene encoding mitochondrial protein	Gutamine metabolic process	3.0
Susd4	Sushi domain containing 4	Unknown	2.9
Slc16a5	Solute carrier family 16 (monocarboxylic acid transporters), member 5	Unknown	2.9
Ccrn4l	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	Rhythmic process	2.9
Lhpp	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Metabolic process	2.7
Neb	Nebulin	Regulation of actin filament length/sarcomere organization	2.6
Got1	Glutamate oxaloacetate transaminase 1, soluble	Oxaloacetate metabolic process/glycerol biosynthetic process/cellular amino acid metabolic process/aspartate metabolic process/aspartate biosynthetic process/biosynthetic process/glutamate catabolic process to aspartate/glutamate catabolic process to 2-oxoglutarate/dicarboxylic acid metabolic process/fatty acid homeostasis	2.6
Sult5a1	Sulfotransferase family 5A, member 1	Unknown	2.6
Hapln1	Hyaluronan and proteoglycan link protein 1	Cell adhesion	2.5
Mt2	Metallothionein 2	Cellular zinc ion homeostasis/nitric oxide mediated signal transduction/detoxification of copper ion	2.5
Mt1	Metallothionein 1	Cellular metal ion homeostasis/cellular zinc ion homeostasis/nitric oxide mediated signal transduction/detoxification of copper ion	2.4
Slc3a1	Solute carrier family 3, member 1	Amino acid transport	2.4
Trdn	Triadin	Cellular calcium ion homeostasis/regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum/negative regulation of calcium ion transport via store-operated calcium channel activity	2.4
Bhlhe41	Basic helix-loop-helix family, member e41	Negative regulation of transcription from RNA polymerase Ⅱ promoter/transcription/regulation of transcription, DNA-dependent/circadian rhythm/entrainment of circadian clock/regulation of transcription	2.3
Usp2	Ubiquitin specific peptidase 2, transcript variant 3	Ubiquitin-dependent protein catabolic process	2.3
Derl3	Der1-like domain family, member 3	Unknown	2.3
Mrap2	Melanocortin 2 receptor accessory protein 2, transcript variant 1	Unknown	2.2
Ncam2	Neural cell adhesion molecule 2, transcript variant 1	Cell adhesion	2.2
S1pr5	Sphingosine-1-phosphate receptor 5	Signal transduction/G-protein coupled receptor protein signaling pathway	2.2
1810046K07Rik	RIKEN cDNA 1810046K07 gene	Unknown	2.2
Nrg4	Neuregulin 4	Unknown	2.2

Gas2	Growth arrest specific 2	Apoptosis/cell cycle/cell cycle arrest/regulation of cell shape	2.2
Ttc39b	Tetratricopeptide repeat domain 39B	Unknown	2.2
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	Steroid biosynthetic process/glucocorticoid biosynthetic process/oxidation reduction	2.1
Creld2	cysteine-rich with EGF-like domains 2	Unknown	2.1
Upp2	Uridine phosphorylase 2	Nucleoside metabolic process/nucleotide catabolic process	2.1
Ar	Androgen receptor	*In utero* embryonic development/transcription/regulation of transcription, DNA-dependent/regulation of transcription from RNA polymerase Ⅱ promoter/male gonad development/cellular process/regulation of gene expression/male somatic sex determination/androgen receptor signaling pathway/androgen receptor signaling pathway/positive regulation of estrogen receptor signaling pathway/positive regulation of MAPKKK cascade/positive regulation of insulin-like growth factor receptor signaling pathway	2.1
Gm10419	Lung RCB-0558 LLC cDNA, RIKEN full-length enriched library, clone: G730014J15 product: Hypothetical protein	Unknown	2.1
Sdf2l1	Stromal cell-derived factor 2-like 1	Unknown	2.0
Trdn	Triadin	Cellular calcium ion homeostasis/regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum/negative regulation of calcium ion transport *via* store-operated calcium channel activity	2.0
Antxr2	Anthrax toxin receptor 2	Unknown	2.0

S. cerevisiae: *Saccharomyces cerevisiae*.

Table 3 Pathway analysis of differentially expressed genes in the Gcgr$^{-/-}$ mouse liver

	Glucose homeostasis	Inflammation	Cell proliferation	Metabolism
Underexpressed in Gcgr$^{-/-}$ mouse liver	Nnmt, Got1, Sds, Pck1		Gas2	Slc10a2, Ntrk2, Gls2, Lhpp, Sult5a1, Mt2, Mt1, Slc3a1, Trdn, Usp2, Cyp17a1, Upp2
Overexpressed in Gcgr$^{-/-}$ mouse liver		Il1b, Cxcl13, Tgtp1, Cxcl9, Defb1, Vtcn1, H2-Ab1, Spon2, Il2rg	Cdkn1a, Igfbp1, Chac1, Cav1, Inhba, Gadd45b, Rassf4, Socs2, Meig1	Serpina7, Cpt1b, Slc34a2, Fabp5, Lpl, Acaca, Asns

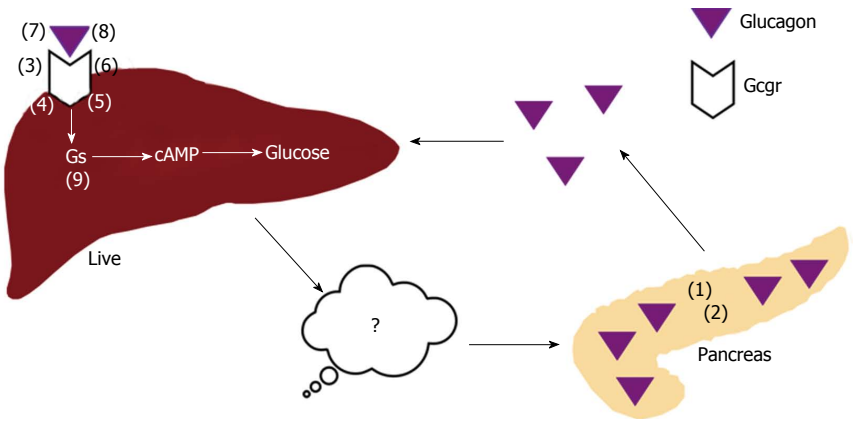

Figure 2 Schematic drawing of regulation of pancreatic α cell number by a humoral liver factor. The numbers indicate specific ways to disrupt glucagon signaling. (1) Glucagon deletion; (2) PC2 deletion; (3) Glucagon receptor (Gcgr) global deletion; (4) Gcgr liver-specific deletion; (5) Gcgr inactivating mutation; (6) Gcgr antisense RNA; (7) Gcgr antagonists; (8) Gcgr antibodies; and (9) Gsa liver-specific deletion. See text for details.

receptor for the inhibitor (leptin receptor). Analogously, the Gcgr$^{-/-}$ mice liver could produce a stimulator or lack an inhibitor of α cell mass. Whether the liver factor is a stimulator or an inhibitor in nature is critical in guiding the search for the identity of the liver factor that causes ACH. As parabiosis is technically challenging, it probably should be used as a last resort. Alternative approaches such as primary pancreatic islet culture[52,53] may be used first to resolve the stimulator/inhibitor question and later used as a high-throughput model for identifying the factor. For example, if a 10:1 mixture of wildtype and Gcgr$^{-/-}$ serum stimulates α cell proliferation of the wildtype islets, then it is likely that the Gcgr$^{-/-}$ mice have a stimulator of α cell mass. Another potential systems approach is to compare the liver gene expression and metabolic profile of multiple animal models of reactive ACH. As all the models develop reactive ACH, any differentially expressed genes unlikely encode the liver

factor and metabolites of various levels unlikely cause ACH, thus greatly narrowing down the list of candidate genes or metabolites. It is also important to point out that other subtypes of ACH exist and not all ACH is associated with glucagon receptor mutation[22,54].

CONCLUSION

Pancreatic ACH is a precursor lesion that gives rise to PNETs. Reactive ACH is associated with hyperglucagonemia and invariably evolves into PNETs in both humans and animal models. The glucagon receptor knockout ($Gcgr^{-/-}$) mice are one of the murine model of reactive ACH and current research has shown that the liver produces a factor that regulates pancreatic α cell mass. Liver gene expression arrays and metabolic profiling suggest a number of potential candidates for the novel liver hormone but none of them so far tested has been confirmed. As understanding the physiological, cellular, and molecular mechanisms underlying reactive ACH pathogenesis is important to the prevention and treatment of PNETs, the search for the elusive liver factor is worthwhile but may require a substantial effort to find it.

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REVIEW

Magnetic resonance imaging biomarkers of gastrointestinal motor function and fluid distribution

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Abstract

Magnetic resonance imaging (MRI) is a well established technique that has revolutionized diagnostic radiology. Until recently, the impact that MRI has had in the assessment of gastrointestinal motor function and bowel fluid distribution in health and in disease has been more limited, despite the novel insights that MRI can provide along the entire gastrointestinal tract. MRI biomarkers include intestinal motility indices, small bowel water content and whole gut transit time. The present review discusses new developments and applications of MRI in the upper gastrointestinal tract, the small bowel and the colon reported in the literature in the last 5 years.

Key words: Magnetic resonance imaging; Stomach; Small bowel; Colon; Motility

Core tip: Magnetic resonance imaging (MRI) of gastrointestinal motor function and fluids distribution is coming of age, with a range of MRI biomarkers that can be measured non-invasively. The novel MRI biomarkers include intestinal motility indexes, the small bowel water content and whole gut transit time. Future research directions will focus on small and large bowel motility and on gut transit. Further validation of the methods and automation of data analysis will finally translate the MRI biomarkers into clinical routine.

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INTRODUCTION

The first demonstrations of the use of dynamic, serial and cine magnetic resonance imaging (MRI) to investigate organ motor function and fluid distribution in the gastrointestinal (GI) tract were reported nearly three decades ago[1,2]. For a long period of time this niche field was explored in a handful of MRI research laboratories and dedicated researchers that put up with the very laborious and lengthy manual data processing, often carried out image by image. Recent advances in imaging methods and data analysis tools are now bringing MRI-based assessments of GI function and fluids into the clinical arena. The number of MRI biomarkers, as indicators of GI function that can be objectively measured, has broadened (Table 1). MRI is often perceived as an expensive technique; however the cost of a short MRI scan compares favorably with more invasive procedures such as, for example, manometric intubation. This review focuses only on the last 5 years of relevant literature using MRI to study gastrointestinal motor function and bowel fluid distribution in the upper GI tract, the small bowel and the colon in health and in disease. Previous years were covered by preceding reviews[3-5].

MRI OF GASTROINTESTINAL MOTOR FUNCTION

Esophagus

The dynamic of swallowing has been investigated with high temporal resolution MRI, providing functional information[6-8]. The images nicely delineate the motor action, and further work to validate these observations and establish clinical indications for "MR esophagography" would be welcome. One study showed a morpho-functional application to the study of achalasia[9] and another showed motility disturbances in some patients after Nissen fundoplication[10]. Gastroesophageal reflux was elegantly visualized using MRI and concomitant high resolution manometry[11] (Figure 1) with a view to improve understanding of reflux suppression by a raft-forming alginate, compared to a different antacid formulation. The same group provided a detailed biophysical analysis of the function and structure of the gastro-esophageal junction[12-14] hypothesizing that components of a "flap valve" contribute to reflux protection, and that this is impaired in patients with gastro esophageal reflux disease. These are unprecedented biomechanical insights into the function of the upper GI tract.

Stomach

There has been continuing interest in the effect of manipulating the physical properties of food components on gastric motor function and appetite. Aerated foams were imaged for the first time *in vivo* demonstrating their effect on increasing gastric volumes and reducing appetite compared to isocaloric, non-aerated beverages[15]. It was also shown that fat emulsions of varying droplet size can modulate gastric emptying[16,17]. The data processing required to monitor gastric volumes and emptying can still be a burden. Developments were made in modeling the emptying curves including gastric secretion[18,19] and in automating the analysis[19-21], with a view to creating a protocol that would be acceptable in clinical practice. Gastric motility was evaluated by simple review of cine MRI series across the stomach after laparoscopic sleeve gastrectomy[22]. The sleeve was found to have little peristaltic function whilst the antrum showed accelerated propulsion. Comparison between manual and automated analysis of gastric motility[23], concluded that the semi-automated procedure for segmentation had comparable accuracy and much better efficiency than the manual method.

Small bowel

The MRI assessment of small bowel motility is the field that has seen some of the most interesting developments over the last 5 years. A number of publications reported developments towards increased automation of analysis and quantitation of small bowel motility biomarkers. The task is still challenging. Good bowel distention is generally required; this is achieved by either infusing a large amount of liquid contrast directly in the small bowel using a catheter (MR enteroclysis) or by ingesting it [magnetic resonance enterography (MRE)]. MRE has been more popular because it is less demanding on both staff and patients. There is however little consensus. Based on local preferences, different contrast media, prone or supine position as well as different acquisition protocols and analysis strategies are used.

In terms of data acquisition, different MRI protocols have been proposed. Qualitatively, many MRI units nowadays add a short cine sequence to small bowel protocols, before injection of spasmolytics, for an overall visual assessment or operator's grading of motility[24,25]. Robust biomarkers however require objective quantitation and their translation requires improvements in data processing. There are two distinct schools of thoughts: One prefers breath-hold acquisitions whilst the other favors acquiring data for longer periods of time, free-breathing. The former minimizes diaphragmatic displacement thus making the data analysis easier. Multiple breath-holds can be acquired to sample motility for longer periods. Displacement of the small bowel by abdominal or diaphragmatic movement can affect the analysis during prolonged observation; this was evaluated in the prone position finding that craniocaudal displacement is predominant but the amplitude of the displacement is modest[26]. The second school of thought seeks to acquire for longer periods of time with the patient breathing freely and gently. In this case respiratory motion affects the quantitation of motility substantially and techniques are needed to correct for this in the time series before analysis. Robust Data Decomposition Registration (RRDR)[27] was used as a pre-processing step to remove respiratory motion; after

Table 1 Magnetic resonance imaging biomarkers of gastrointestinal motor function and fluid distribution

Biomarker	Method	Ref.
Gastric emptying	Time courses of gastric volumes, ROI analysis	[18-21]
Gastric secretion volume	T1 mapping, dilution of a meal labeled with gadolinium contrast agent	[19,67]
Gastric motility	Cine-MRI	[23]
Small bowel motility	Cine-MRI, image registration, standard deviation of the Jacobian	[28,29]
Small bowel water content	Heavily T2 weighted imaging, ROI analysis using calibrated threshold	[61]
Oro-cecal transit time	Arrival of the head of a meal in the cecum	[65]
Colonic volumes	ROI analysis	[58,59]
Colon water content	Heavily T2 weighted imaging, ROI analysis using calibrated threshold	[74]
Colon motility	Cine-MRI, image registration, line ROI analysis	[28]
Whole gut transit	T1-weighted imaging, capsules filled with water and gadolinium contrast agent	[65]
Colonic chyme relaxometry	T1 and T2 measurements	[61,74]

MRI: Magnetic resonance imaging; ROI: Region of interest.

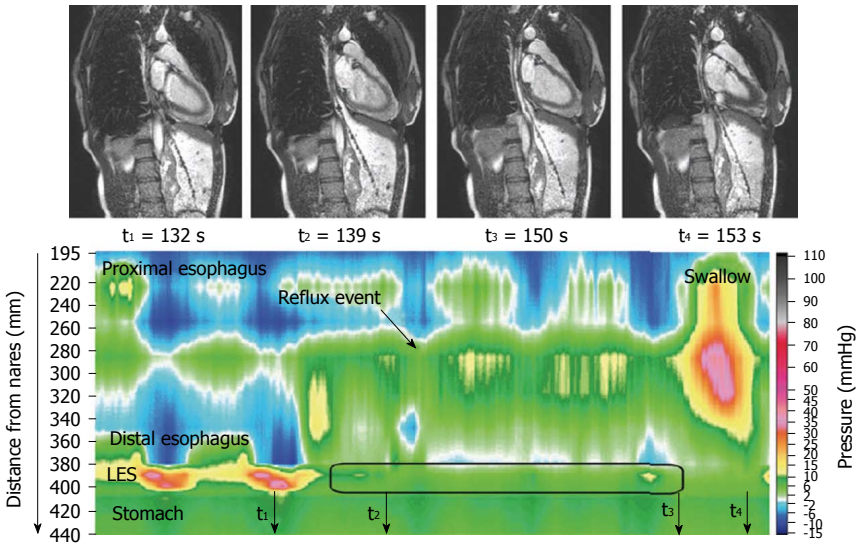

Figure 1 Concurrent high-resolution manometry and magnetic resonance imaging. Representative image demonstrates concurrent high-resolution manometry and magnetic resonance imaging detection of reflux. Note that shortening of the esophagus in the dynamic magnetic resonance images appears to draw the proximal stomach upwards relative to the catheter (above). Reproduced with permission from ref. [11].

this step global small bowel motility[28] was determined using an optic flow registration method[29]. The motility biomarker is based on the standard deviation of the Jacobian calculated from the displacement fields of the image pixels. This biomarker is based on the pixel intensity changes that the software uses to derive the registration parameters; hence it is not exactly anchored "biomechanically" to the bowel walls. On the other hand, the method provides an elegant and operator-independent assessment of global motility from long, free breathing time-series and yields motility maps that are easy to interpret (Figure 2). Another automated approach based on the optic flow registration technique was implemented, without the dual registration pre-step, in studies in IBD patients[30,31]. An alternative MRI approach to monitor motility is the continuous tagging, as is common in cardiac MRI. A global tagging motility index biomarker was used[32] with the motility analysis subdivided in low, medium and high frequency bands[33]. The index was able to detect a decrease in motility due to intravenous anti-peristaltic agent. The tagging method is region of interest (ROI)-independent. Tagging may also depend less on bowel distension, as suggested by the authors suggest[32].

In terms of data analysis, there was a limited use of visual, consensus analysis[34], mean change in signal amplitude[35] and manual luminal caliber measurements[36]. Software assisted methods were applied to both breath-hold and free-breathing acquisitions[23,37,38] and performed better than manual measurements[39]. The choice of intra-segmental location for the software-assisted analysis did not influence substantially the measurements substantially[40]. Region of interest analysis of small bowel motility showed however inter-segmental variation and modest repeatability[41], which would favor global, operator independent methods[42]. The frequency band analysis of continuously tagged images was also assessed automatically[33].

The MRI assessment of motility has found interesting applications in Crohn's disease (CD), a particularly vulnerable population. These patients are likely to undergo serial imaging examination over the course

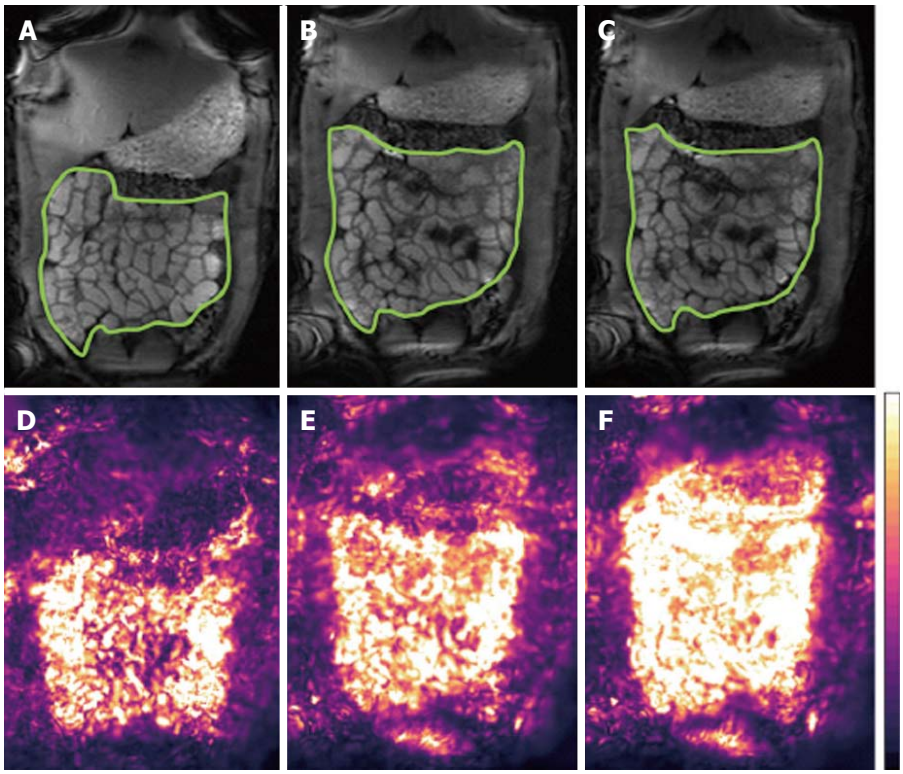

Figure 2 Small bowel motility maps. Example of small bowel regions (contoured) in the upper panel and motility biomarker maps in the lower panel. Respectively: breath-hold ground truth (A and D), dual-registration of abdominal motion (B and E) and free breathing optical flow registration alone (C and F), respectively. Respiratory motion compensation is visible as reduced motility in the transverse colon closest to the diaphragm and systemically over the small bowel. The effect of robust data decomposition registration is less apparent in the lower bowel further from the diaphragm where the effects of free breathing are less pronounced. The color coding I the motility maps shows black as lower motility and white as higher. Reprinted with permission from ref. [28].

of their treatment and the cumulative radiation dose from repeated computed tomography is undesirable[43]. Reduced motility was associated with small bowel segments affected by CD[24], correlating well with histopathology[44] and inflammatory markers in the blood and stools[45]. Notably the MRI motility biomarker reflected disease activity. Motility scores were associated negatively with disease activity score[46,47], using a multivariate analysis based on mural thickness, mural T2 signal, perimural T2 signal and enhancement[48]. Another finding of great interest is the demonstration that small bowel motility is not only impaired at the site of the lesion but also proximally[49-51]. The availability of cine MRE images was shown to aid the reader's evaluation of questionable segments in a less ordinary CD exam protocol without the use of anti-peristaltic agents[52].

Beyond specific CD applications, cine MRI of small bowel motility was used to compare intravenous and intramuscular delivery routes for anti-peristaltic agents[53]. The data showed that intravenous administration had a faster and more reliable onset, whilst a combination of different agents and delivery routes provided early onset and high degree, sustained spasmolysis. The effectiveness of sublingual hyoscyamine sulphate as an alternative to antiperistaltic intravenous agents was also investigated using cine MRI[54]. The treatment effect of the sublingual agent was modest. The oral glucose tolerance test was shown to accelerate intestinal motility after laparoscopic sleeve gastrectomy[25]. Another interesting application of cine MRI was in chronic intestinal pseudo-obstruction (CIPO), showing contractility impairments in the CIPO patients compared to healthy volunteers and patients with irritable bowel syndrome[55]. It is worth noting that MRI of small bowel motility has also found some applications in animal models[56,57] although those are beyond the scope of this review. MRI was also used to study postprandial colon volumes as another biomarker of function[58]. Manual colon segmentation is lengthy and methods to semi-automate the processing have been proposed recently[59].

Despite the lack of standardization and the need for some further validation, the emerging biomarkers of small bowel motility are very promising and the body of recent work demonstrates that cine MRI of small bowel motility is coming of age. The data acquisition can translate to the clinics relatively easily. The high-end image registration and data processing methods may however require implementation in the scanner viewing platforms or dedicated cloud computing services for the technique to move into routine use.

Colon

Despite the flourishing of MRI publications on small bowel motility, so far little attention has been given

to colonic motility. One possible reason for this is that colonic motility is inherently erratic so that an observation based on a single breath-hold cine slab may not be very informative. A longer acquisition time of a cine MRI sequence would characterize motility better. However, the same respiratory motion problems detailed above for the small bowel will affect the data.

The published studies used a variety of approaches. Visual inspection of cine MRI stacks showed reduced or absent peristalsis in involved colonic segments of 3 patients with ulcerative colitis, compared to other bowel segments[34]. In one elegant study bisacodyl instillation was used to induce high amplitude propagated pressure waves in the (cleansed) descending colon of 10 healthy volunteers and motility was monitored by concomitant MRI and manometry[60]. Three perpendicular imaging planes were acquired at 4 s intervals at baseline and for 24 min post bisacodyl instillation. The MRI images in each plane were played as a cine loop identifying changes of 50% in the largest diameter of the haustras. Eleven of these larger amplitude contractions were detected and these had an excellent 100% correlation with the manometry readings.

In a different study a subjective colonic motility index score was assessed by an operator in response to an oral polyethylene glycol (PEG) stimulus that distended the ascending colon and stimulated motility in healthy volunteers[61]. A single sagittal slice was acquired every second for 2 min of free breathing. No motion correction was applied and the operator inspected the data by dividing the ascending colon in three regions, estimating for how long each region showed contractility. This applied to any visible contractility not just high amplitude propagated waves. Using this relatively basic method the authors showed a marked increase in motility upon ingestion of PEG and that the increase was dose-dependent.

More quantitative approaches can clearly benefit from the registration of abdominal motion as discussed for the small bowel. A recent study applied the optic flow and RRDR dual-registration method to MRI data from the ascending colon of 6 healthy volunteers who ingested an oral PEG stimulus[28]. A single sagittal slice was again acquired every second for 2 min of free breathing. The study then compared simple line ROIs analysis results with and without application of the motion correction and showed the importance of correcting for abdominal motion to remove ambiguity. Optic flow methods were also used to quantify effectively hypomotility of colonic segments affected by CD using the static images as guide to define regions of interest in global motility maps[30].

Work this area is likely to continue in the next few years and the focus for new developments will expand from the small bowel towards MRI of colonic motility.

Flow and transit

Bowel luminal flow has been overlooked whilst MRI of gastrointestinal transit has been the subject of a few new technical development studies. Three studies by Hahn *et al*[62] sought to use ^{19}F imaging and MRI "transit capsule markers". This is an interesting approach as there is basically no endogenous fluorine MRI signal in the human body, so any signal detected can be attributed to the capsules. Moreover the ^{19}F nucleus has particularly good MRI visibility with 100% natural abundance and a gyromagnetic ratio close to the one of the hydrogen proton. The authors were able to show simultaneous, real-time tracking of one and two capsules in the GI tract of two healthy volunteers using ^{19}F projection imaging superimposed to a proton anatomical reference[62] (Figure 3). In subsequent studies the "3D golden angle radial projection" ^{19}F imaging was deployed[63]. Using this acquisition they tracked capsules either embedded in a naso-gastric catheter (to enable tracking of the catheter) or ingested (to track the transit of the capsules in the GI tract) by one healthy volunteer. The ^{19}F MRI catheter tracking methodology was further improved which allowed real time visualization and manipulation of the catheter[64]. The idea of using ^{19}F to monitor GI transit is elegant; however there are significant barriers to translation including the need to use high field ($\geqslant$ 3T), multinuclear transmit and receive hardware and a dedicated abdominal ^{19}F transmit/receive coil, of which at the moment there are only few worldwide. The capsules are also relatively large (12 mm × 7 mm) and so unlikely to empty from the fed stomach. They are more likely to remain within the stomach until expelled by the migrating motor complex which will not develop until the fasting state is reached. Thus propulsion of these capsules along the GI tract is unlikely to mirror physiological transit of food. A different approach has been to use the proton MRI and MRI "transit capsule markers" filled with water doped with trace amounts of gadolinium contrast agent. Measurement of whole gut transit based on ingestion of 5 such markers and T1-weighted imaging was validated against standard radiopaque marker X-ray methods with repeated studies in 21 healthy volunteers[65]. The MRI method performed well against X-ray methodology and does not require high field or additional hardware. However the capsules are again relatively large (20 mm × 7 mm) and gastric sieving is likely to retain them during the fed state so they will only leave the stomach after the food has left. Furthermore their signal could be confused with high T1 food residue particularly at the terminal ileum/proximal colon. Within the same study, a simple method to measure oro-cecal transit time (OCTT) based on imaging the arrival of the "head of a meal" in the cecum was also evaluated against concomitant standard lactose ureide ^{13}C breath test[65]. Correlation between the two methods was weak. Another major limitation of this MRI method is the need to continue imaging at intervals until the arrival of the "head of the meal" in the cecum is detected. This limits the time resolution of OCTT to the sampling frequency which is unsatisfactory. Furthermore the repeated scanning until detection is achieved would make its routine use expensive. Another study sought to evaluate OCTT by

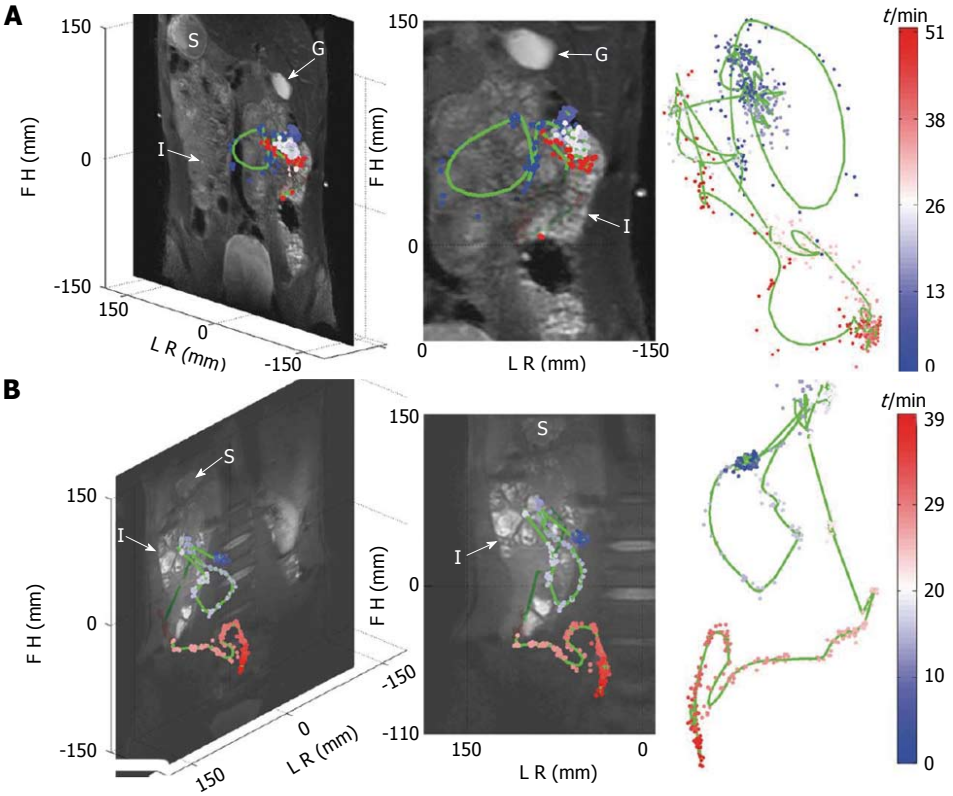

Figure 3 ^{19}F magnetic resonance imaging tracking of transit marker capsule in two healthy subjects. The panel shows anatomical reference images, ^{19}F capsules positions and the fitted intestinal course for subjects (A) and (B). The labels on the figure denote the stomach (S), gall bladder (G) and small intestine (I) and the time course of the two capsules is color coded. Reprinted with permission from ref. [62]. LR: Left-right; FH: Feet-head.

similar MRI methods comparing the results to concomitant standard lactulose hydrogen breath test[66]. The passing of the lactulose fluid bolus through the small bowel was followed visually on T2 weighted images until its arrival in the cecum was detected.

These studies show an increasing interest in developing non invasive MRI biomarkers for both oro-cecal and whole gut transit. Further work is needed to improve such methods and make them more physiological if they are to translate to the clinics effectively.

MRI OF GASTROINTESTINAL FLUID DISTRIBUTION

Stomach

The investigation of fluids in the upper GI was predominantly focused on gastric secretion as measured by T1 mapping of a test meal doped with traces of a Gd-based contrast agent[19,67]. This showed a layer above the liquid meal in the stomach containing a lower concentration of contrast agent[68]. This is consistent with the concept of the "acid pocket" and could provide a target for gastroesophageal reflux treatments. Another study assessed the effect of pharmacologically enhanced gastric secretion on ^{13}C-acetate breath test for gastric emptying[69]. There was new interest from the point of view of pharmaceutical sciences and drug dissolution. Two new studies investigated gastric fluid content under the standard fasting[70] and fed oral dosage form conditions[71] with a view to improving *in vitro/in vivo* correlation of drug dissolution modeling.

Small bowel

A number of studies evaluated the fluid content of the small bowel. Some monitored the effect of nutritional interventions[16,17,72,73]. These showed that the effect of physicochemical modifications in food microstructure (such as for example fat emulsion stability and droplet size) can markedly modulate small bowel postprandial fluid inflow. One study demonstrated the effect of a bowel preparation containing polyethylene glycol and electrolytes in generating inflow of fluid in the lumen[61]. By contrast another study showed the ability of a common anti-diarrheal agent, loperamide, to reduce the small bowel water content after a mannitol challenge model of secretory diarrhea[74]. Bowel fluid was also shown to be increased by an essential amino acid[75]. Other MRI studies showed that experimental stress reduced small bowel water content[76]. The effect of poorly absorbed and non absorbable carbohydrates on bowel fluid inflow and accumulation was also studied; these included fructose[77] (Figure 4) and lactulose[78]. The presence of separate small water pockets in the fasting small bowel was confirmed and the distribution and volume of the bowel pockets measured before and after ingestion of the standard fasting drug testing dose of 240 mL of water[70] with the same pharmaceutical sciences rationale as described above. The main finding

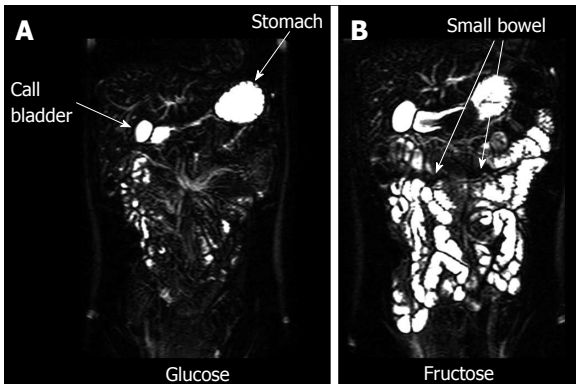

Figure 4 Small bowel water imaging. Representative example of coronal images of the small bowel water from a single volunteer acquired 75 min after drinking 40 g of glucose (A) or 40 g fructose (B) in 500 mL water. Glucose is rapidly absorbed so the small bowel has very little water in it despite the large drink. Conversely fructose is poorly absorbed and osmotically active as shown by the large amount of water in the small bowel. Adapted with authors' own copyright from ref. [77].

was that the small bowel water pockets are discontinuous and their number and volume is small.

Colon

Two studies addressed colon fluid distribution using MRI. One study used an oral mannitol challenge and showed inflow of water from the small bowel into the ascending colon[74], quantifying the amount of freely mobile water in the ascending colon using similar methods as those used for the small bowel. The study found that there was only a small amount of freely mobile water detectable in the ascending colon. T2 relaxometry was also used in that study to characterize physicochemical changes in the chyme upon arrival of the fluid bolus, which showed an increase in T2 reflecting increased fluid mobility in the chyme. The other study showed that ingestion of a bowel preparation containing polyethylene glycol and electrolytes reached the colon rapidly increasing its size two-fold[61]. The study also used T1 relaxometry to characterize physicochemical changes in the chyme upon arrival of the fluid bolus. The relaxation time T1 of the ascending colon contents increased upon arrival of the fluid in the chyme as expected. Given the growing interest in bowel fluid dynamics and the work conducted so far more proximally, one can predict that MRI of colonic fluids will be an expanding field in the near future.

CONCLUSION

MRI of gastrointestinal function is coming of age. The development of more automated analysis methods will aid translation into clinical routine although further work on validating the MRI biomarkers is needed. The novel insights provided on bowel fluid volumes and distribution will improve understanding of disease and predictive models of drug dissolution. Further trials are needed to prove the value of the MRI biomarkers in clinical practice.

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REVIEW

Eosinophilic esophagitis: From pathophysiology to treatment

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Abstract

Eosinophilic esophagitis (EoE) is a chronic immune disease, characterized by a dense eosinophilic infiltrate in the esophagus, leading to bolus impaction and reflux-like symptoms. Traditionally considered a pediatric disease, the number of adult patients with EoE is continuously increasing, with a relatively higher incidence in western countries. Dysphagia and food impaction represent the main symptoms complained by patients, but gastroesophageal reflux-like symptoms may also be present. Esophageal biopsies are mandatory for the diagnosis of EoE, though clinical manifestations and proton pump inhibitors responsiveness must be taken into consideration. The higher prevalence of EoE in patients suffering from atopic diseases suggests a common background with allergy, however both the etiology and pathophysiology are not completely understood. Elimination diets are considered the first-line therapy in children, but this approach appears less effective in adults patients, who often require steroids; despite medical treatments, EoE is complicated in some cases by esophageal stricture and stenosis, that require additional endoscopic treatments. This review summarizes the evidence on EoE pathophysiology and illustrates the safety and efficacy of the most recent medical and endoscopic treatments.

Key words: Eosinophilic esophagitis; Eotaxin; Immune system; Proton pump inhibitors-responsive eosinophilia; Endoscopic dilation

Core tip: Eosinophilic esophagitis (EoE) is a chronic immune disease, characterized by a dense eosinophilic infiltrate in the esophagus, leading to bolus impaction and reflux-like symptoms. The pathophysiology of this

entity is still unclear, however the involvement of both genetic and immune factors have been suggested. In this review we summarize the evidence on EoE pathophysiology and illustrate the safety and efficacy of the most recent medical and endoscopic treatments.

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INTRODUCTION

The eosinophilic esophagitis (EoE) is a chronic immune disease, characterized by a dense eosinophilic infiltrate into the esophagus. Dysphagia and food impaction episodes are recognized as the main symptoms of EoE in adults, however regurgitation, chest pain and heartburn may also be referred[1].

Considered as a pediatric disease until few years ago, it is now clear that EoE may also occur in adults, especially young Caucasian man[2]. A rapid increase of incidence in the last decades has been registered, however a retrospective study[3] on biopsies collected between 1982 and 1999 revealed that the incidence of EoE appears stable, suggesting that the higher rate of new diagnosis depends, almost in part, on the improved disease recognition. African, West Asian and South American population share a low prevalence rate, but the real incidence of EoE in these countries remains unclear[4,5].

The EoE-symptoms pattern is heterogeneous and although dysphagia and food impaction are frequently reported, patients may also complain of typical and atypical gastroesophageal reflux disease (GERD) symptoms, leading to a delay in the diagnosis.

Endoscopic features, such as esophageal rings, white exudates or plaques, longitudinal furrows, diffuse esophageal narrowing, and mucosal fragility, may help the diagnosis, but histological identification of predominant eosinophilic-inflammation, with more than 15 eosinophils for high power field, represents the main diagnostic criterion for EoE (Figure 1 and Table 1). Nevertheless, the histological evidence of esophageal eosinophilia in a subset of proton pump inhibitors (PPI) responder patients further complicates the diagnosis. Previously considered a GERD-subtype, actually, PPI-responsive esophageal eosinophilia (PPI-REE) is considered a different entity, not distinguishable from EoE[1,6]; in the Figure 2 it is summarized the diagnostic algorithm in case of suspected EoE.

The pathogenesis of EoE is unknown, but it is supposed to be multifactorial with genetic, immunologic and environmental factors being all involved. There is evidence that EoE is more prevalent in patients suffering from food-allergy, rhinitis, asthma or atopic dermatitis[7,8]. Interestingly, all these pathologies share an altered immune response to common antigens, which determinates an aberrant Th_2-response, and, hence, the uncontrolled activation of eosinophils, mast cells and basophils[9-13].

Specific allergy testing, or empiric elimination diets represent the first line therapy in children with EoE[1,14]. Topical corticosteroids are considered the mainstay of therapy for adult patients[1,15], while systemic steroids are reserved to patients with persistent eosinophilia[1]. Besides its central role in the diagnosis of EoE, endoscopy has also of great impact on the treatment of EoE fibrotic complications[1,16].

This review summarizes the most recent evidence on the pathogenesis of EoE, focusing on the role of genetic and immunologic factors and illustrates the safety and efficacy of the most recent medical and endoscopic treatments.

PATHOPHYSIOLOGY

Genetic factors

The higher risk of EoE in familiars of affected patients supports the hypothesis of genetic predisposition. The latest familial study[17] estimated that 2.4% of proband siblings' also had EoE, with a 40-folds higher risk, than general population.

The incidence of EoE is higher in monozygotic twins (41%), but the observation that the disease also occurs in 21% of dizygotic twins suggests a role for environmental factors, especially in the early-life[17]; by using a complex statistical model it has been estimated that the contribution to the familiar risk depends by hereditability and exposure to common environment for 14.5% and 80%, respectively[17].

Three different approaches have been used to identify the genes involved in EoE predisposition: The association with Mendelian syndromes, the search for a specific gene and the genome-wide association studies[18] (Table 2).

An increased prevalence of EoE has been reported in patients affected by hypermobile connective tissue diseases, like Marfan, Ehler-Danlos and Loeys-Dietz Syndromes; interestingly, these pathologies are characterized by a defective transforming growth factor (TGF)-β pathway, and the observation that this factor is increased in the esophagus of both syndromic and not EoE likely supports its causative role[19-21]. A major risk of EoE has been also described in some pro-allergenic Mendelian diseases, like the "Iper-IgE syndrome"[18] and an autosomal dominant disease belong to Mast-cell Activation syndromes, characterized by high levels of mast cell tryptase, this association strongly suggests the pathogenic association between EoE and atopic diseases[22]. This concept is further supported by the association between EoE and a rare syndrome characterized by severe atopic dermatitis, multiple allergies and metabolic syndrome (SAM); this syndrome is characterized by a mutation of desmoglein-1 gene's[18,23], whose expression

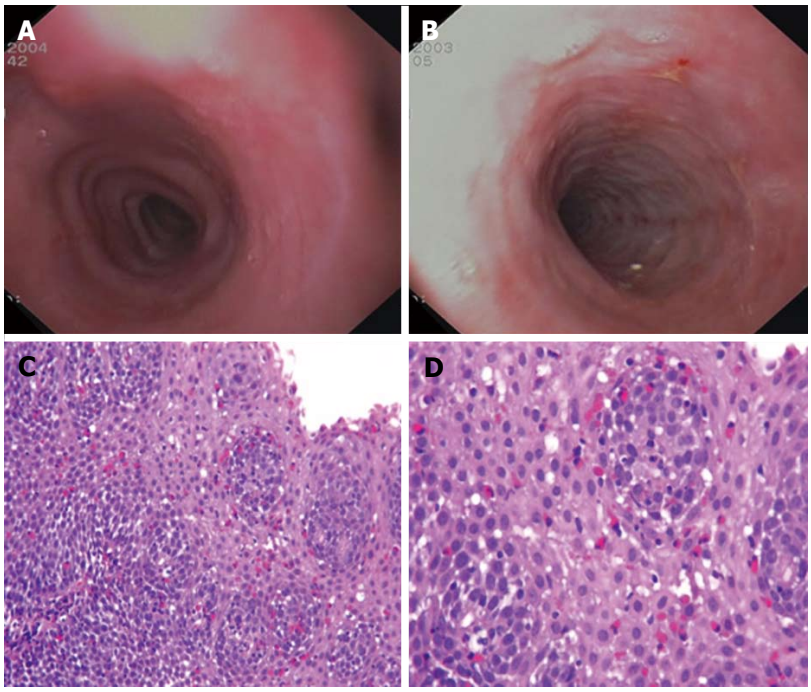

Figure 1 Endoscopic and microscopic findings in eosinophilic esophagitis. A: Esophageal rings; B: White exudates, longitudinal furrows and mucosal fragility; C and D: Esophageal mucosa infiltrated by several eosinophils (red cells) (C: Original magnification HE 150 ×; D: HE 400 ×).

Table 1 Endoscopic and histological features of eosinophilic esophagitis

Endoscopic features
Esophageal rings
White exudates or plaques
Longitudinal furrows
Diffuse esophageal narrowing
Mucosal fragility
Histological features
Eosinophilic infiltration (≥ 15 eos/hpf)
Eosinophilic degranulation
Basal zone hyperplasia
Eosinophilic micro-abscesses
Spongiosis or dilated intercellular spaces
Intramucosal lymphocytes

is significantly reduced in idiopathic EoE[24].

Candidate-gene identification studies allowed identifying the putative factors associated with EoE in non-Mendelian syndromes. A single-nucleotide polymorphism (SNP) in the gene CCL26, encoding for the eotaxin-3, was associated with EoE[25]. This protein is overexpressed in esophageal epithelial cells of affected patients, and plays an important role in the chemotaxis of eosinophils[26,27]. Similarly, a SNP in the gene encoding for the filagrin, a structural membrane protein involved in epithelial cells-extracellular matrix interaction has also been identified[18]. Several genes were found to be associated with EoE by genoma wide association studies. In one of the most recent report[28] a significant association between a locus on 5q22, encoding for the cytokine thymic stromal limphopoietin (TSLP) and EoE was reported. Although the specific mechanism remains, yet, to be clarified, TSLP has been demonstrated to have a pivotal role in the activation of basophils in human and animal models of EoE[29,30]. In the same study[28] also the *CAPN14* gene, encoding for the calpain subfamily of proteolytic systems, was identified; more precisely this protein, that is specifically expressed by epithelial cells of the esophagus, is activated by interleukin (IL)-13 and participates to inflammatory process[28].

Immune system factors

The EoE is characterized by a prevalent eosinophilic infiltrate in the lamina propria and submucosa of the esophagus. The precise mechanisms of such localized inflammatory reactions are not recognized yet, but it is suggested that different cytokines are involved in the maturation and migration of eosinophils. In particular, IL-5, IL-13 and granulocyte-macrophage colony-stimulating factor are produced by different cell types, included esophageal epithelial cells, after an appropriate stimulation by the antigen-presenting cells (APCs)[31]. As shown in Figure 3, the evidence that in EoE patients there is desmoglein-1-dependent altered barrier function[23] have led some authors to hypothesize that the increased permeability of esophageal epithelium could facilitate the passage of different antigens, that causes the activation of APCs and invariant natural killer T-cells. These cells, if properly stimulated, are able to prime a Th_2 immune response, by the production of IL-13 and IL-4[32]. However, whether the barrier impairment represents a primum movens, or an epiphenomenon in the context of the eosinophilic-inflammation remains unclear. Sherrill *et al*[33] have demonstrated that esophageal epithelial cells express toll-like receptors, whose antigens-mediated activation, through the production of IL-5 and IL-13[34], is able to trigger a Th_2-

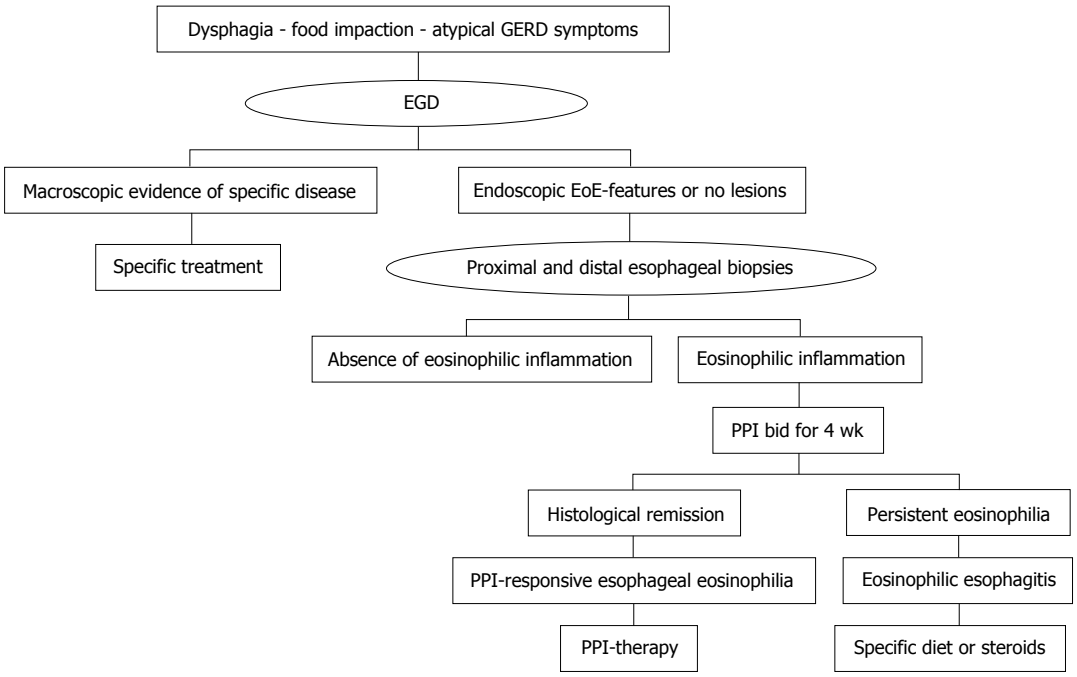

Figure 2 Diagnostic flow-chart of eosinophilic esophagitis. GERD: Gastroesophageal reflux disease; EoE: Eosinophilic esophagitis; PPI: Proton pump inhibitors; EGD: Esophagogastroduodenoscopy.

Table 2 Genetic factors involved in eosinophilic esophagitis

Genes	Encoded protein	Mechanism of action	Ref.
Mendelian syndromes			
FBN1 (Marfan syndrome)	Fibrillin	Alteration of TGF-β pathway	[19]
COL (Elher-Danlos syndrome)	Collagen	Alteration of TGF-β pathway	[19]
TGFBR (Loeys-Dietz syndrome)	TGF-β-promoters	Alteration of TGF-β pathway	[21]
STAT3 (Iper-IgE syndrome)	Transcription activator 3	Aberrant cytokines production	[18]
DSG1 (SAM)	Desmoglein1	Loss of cell-cell adhesion	[23]
EoE-associated genetic variants			
CCL26	Eotaxin-3	Eosinophilic chemo-attraction	[25]
FLG	Filaggrin	Epithelium-ECM interaction	[18]
TSLP (5q22)	TSLP	Basophils chemo-attraction	[28,29]
CAPN14	Calpain 14	Proteolytic effects	[28]

TGF: Transforming growth factor; ECM: Extracellular matrix; SAM: Severe atopic dermatitis, multiple allergies and metabolic syndrome.

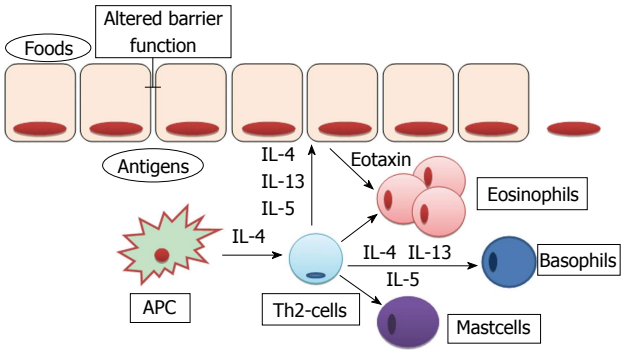

Figure 3 Pathophysiological mechanism involved in eosinophilic infiltration of esophageal mucosa. IL: Interleukin; APC: Antigen-presenting cell.

response with the production of other cytokines and the proliferation of eosinophils, T-cells, mast cells and basophils[31].

The increased release of these cytokines in both the esophagus and the blood of EoE patients has been demonstrated in different studies[35,36], and, although need to be further clarified, their role in the pathogenesis of EoE appears to be fundamental.

IL-4 and IL-13 are able to prolong the T-cell survival and to increase eosinophilic migration through the release of Eotaxin-3 and TSLP by epithelial cells[37]; recently, Zhu *et al*[38] found that IL-15 is also enhanced in EoE and, most interestingly, they demonstrated that IL-15 receptor deficient mice are protected from EoE, but not from other allergic diseases, supporting a specific role of IL-15 in the "allergic-pathway" of esophagus. Although the eosinophilic infiltration represents the main characteristic, it is of relevance that other immune cells, like basophils and mast cells, are also involved in the

pathogenesis of EoE and they likely contribute to the amplification of the esophageal inflammatory response and mucosal damage[18,20,29].

Mechanisms of mucosal damage and fibrosis

Food impaction and dysphagia are the main symptoms in patients with EoE and these are a direct consequence of the esophageal mucosa remodeling and fibrosis.

Eosinophils synthetize and release many proteins and mediators, in particular major basic protein (MBP), eosinophil cationic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, TGF-β, IL-13 and platelet-activating factor. Although all these mediators play a key role in the tissue damage and remodeling, the most robust data are about MBP and TGF-β[37].

MBP is able to directly damage epithelial cells, but also to induce mast cells degranulation, increasing the release of proteolytic enzymes, tryptase and chymase, that further participate to the deconstruction of the extracellular matrix[39]; MBP is also able to stimulate the production of fibroblast growth factor-9, leading to fibroblasts proliferation and activation[40]. Similarly, TGF-β induces both fibroblasts activation and contraction, causing their transformation into myo-fibroblasts[41]; in addition at high concentration TGF-β stimulates epithelial cells to assume phenotypical characteristic of fibroblasts, a process named epithelial-mesenchymal transition[42]. These mechanisms act synergistically, determining the altered synthesis of extracellular matrix proteins, such as collagen, tenascin-C and metalloproteinases[43]. In recent studies the periostin, that is highly expressed in the esophagus of EoE patients, has been proposed as a major determinant of extracellular matrix alteration and epithelial barrier function impairment, because of its ability to determine collagen cross-linking and to bind several matrix proteins and integrins[44].

As mentioned above, other immune cells rather than eosinophils participate to the inflammatory response described in the EoE; recently, the ability of epithelial and mesenchymal cells to synthetize and release different molecules has been also suggested to contribute to tissue damage[12,20,45]. In addition, some authors found a correlation between the inflammatory cells infiltrate and enteric neurons alterations, but the role of enteric nervous system impairment in the process of mucosal damage has not been clarified, yet[46].

THERAPEUTIC OPTIONS

The "3 D-approach" summarizes the three major treatments for EoE: Diet, drugs and dilation. Although clinical remission represents a good parameter to evaluate the effectiveness of therapy, the endoscopic and histological response is required. The main endpoint of diet and drug therapies is the resolution of both symptoms and inflammation, however the complete remission is rare, and the relief of esophageal symptoms in association with a significant reduction of mucosal eosinophilia should be considered a good response[1].

Dietary therapy

Specific dietary approaches are considered the first-line therapy in the treatment of EoE in children, due to the lack of adverse effects and the high rate of response. Three different dietary patterns may be used: (1) elemental diet; (2) allergy test-based diet; and (3) empiric elimination diet[47].

The elemental diet demonstrates the high rate of response (almost 90% in children, 70% in adults), with a rapid relief of symptoms associated with histological remission. This diet contemplates the use of amino-acid based liquid formulas for 4-6 wk, followed by the histological evaluation of response. If the remission is achieved, foods are slowly reintroduced, following a strict scheme which contemplates four different food groups, based on their allergenic potential. A single food of each group should be reintroduced every 5-7 d; in absence of symptoms' recurrence, the endoscopic and histological evaluation should be performed before starting the reintroduction of foods from another group. If a specific food determinates esophageal symptoms, it should be excluded and the next food of the same group tested. Despite the high rate of response, this diet is rarely accepted by patients[48,49].

The allergic-test driven diets showed a rate of response of 70% in children, using the combination of both skin prick and patch tests to identify trigger-foods; this dietary pattern allows the elimination of specific foods, enhancing the compliance, however atopy patch test are not universally accepted for food allergy[50,51]. Moreover, given that the rate of response to this diet in adults is lower, this approach is not recommended in this patients.

The empiric diets, instead, are based on the exclusion of the most allergenic foods, such as cow's milk protein, soy, wheat, egg, peanut/tree nut, and fish/shellfish, independently on allergic tests. This dietary therapy has shown a rate of response of almost 70% in both children and adults. However, if the remission is achieved, an endoscopic and histological evaluation should be performed after the reintroduction of each food[52,53].

In conclusion, although the exclusion of trigger-foods allows a long-term remission without drugs, the high cost of such diets, especially aminoacid based-formulas, the poor compliance and the need for multiple endoscopies, represent important disadvantages for dietary therapy.

Corticosteroids

Several studies have demonstrated the efficacy of systemic corticosteroids, such as prednisolone or methyl-prednisolone, in the remission of both symptoms and eosinophilic infiltration, however the recurrence after the tapering is usually observed. Long-term therapy with corticosteroids is limited by the important adverse effects, hence systemic steroids may be used to rapidly induce remission, but a different maintenance therapy has to be set[54]. Topical steroids, in particular fluticasone

and budesonide, have shown a good rate of response, reducing adverse effects also during long-term treatment[55,56]. Due to the lack of specific dispenser, multi-dose inhalers should be used, and patients have to be instructed to swallow, rather than inhale, the product[57]. A viscous compound of budesonide and sucralose, has also been tested, obtaining excellent results[58]. Topical steroids have shown a good safety profile, and are actually considered the first-line therapy, after the PPI trial, in EoE patients. However, some cases of esophageal candidiasis and herpes infections have been described[59,60].

PPI

When the first consensus guidelines for EoE was published in 2007, a physiological 24h-pH-metry and the persistence of symptoms despite PPI therapy were considered major criteria for diagnosis of EoE. In the last years, several studies have shown that almost 40% of patients with clinical, endoscopic and histological features of EoE responds to PPI therapy, independently on the results of 24 h-pH-metry[61,62].

These data questioned the relationship between GERD and EoE, introducing a novel entity, named PPI-REE, in the consensus of 2011[63]. Actually considered more a subtype of EoE, rather than atypical GERD, this pathology is defined by the presence of all hallmarks (clinical, endoscopic and histological) of EoE, associated with a complete (clinical, endoscopic and histological) remission during PPI therapy[1]. Considering that different studies have demonstrated that patients with PPI-REE share the same genetic and phenotypic background of EoE-subjects, nowadays PPI bid is considered the first-line therapy in all patients with EoE features[1,64].

PPI-REE opened a new field of research, focused on the role of reflux in the pathogenesis of esophageal eosinophilia[65]. The hypothesis that gastroesophageal reflux, even if not pathologic, could enhance epithelial barrier dysfunction, allowing the passage of multiple antigens through the mucosa, has been proposed and evaluated. Different studies have demonstrated that gastric reflux is able to enlarge intracellular space, and the increased transit of molecules through esophageal epithelium has also been observed in both GERD and EoE[66,67]. Accordingly, the exposition of several antigens to APC may induce, in predisposed subjects, a cascade of events, triggering a Th_2-response, and, consequently, the eosinophilic infiltration. In this case the therapeutic effects of PPIs, will depend on their ability to favorite the regeneration of epithelial barrier, reducing the antigen exposition and, hence, the amplification of immune response.

According to others, also GERD, as EoE, represents an immune-mediated pathology, in which acid reflux stimulates the release of cytokines, inducing a specific Th_1-response. Therefore, the activation of immune system and the production of toxic mediators determinates mucosal erosions, rather than the direct caustic action of acid[68]. These authors suggest that in "atopic-subjects", the typical response to acid reflux switch to a Th_2-response, causing an EoE-like damage of the esophagus. Therefore, the reduction of immunogenic trigger, by the inhibition of acid secretion, will determinate the resolution of damage. Furthermore, a direct anti-inflammatory effect of PPIs has been recently demonstrated. In particular, different studies have pointed out the reduction of eotaxin 3 and Th_2-mediators, after treatment with PPI in the mucosa of patients with diagnosis of both EoE and PPI-REE. These events have been observed both *in vitro* and *in vivo* experiments, suggesting that PPIs anti-inflammatory action is acid-independent[69-71].

Summarizing, at the baseline, is not possible to discriminate between EoE and PPI-REE, and, although the probability of PPI response in patients with pathologic 24 h-pH-metria is higher, PPIs trial still represents the only instrument to differentiate these entities (Figure 2)[72,73].

Other pharmacological treatments

Given the association with other atopic diseases, leukotriene antagonist and mast cells stabilizers, such as montelukast and cromolym, have been tested in the treatment of EoE, however all studies showed a poor efficacy of these drugs in both symptomatic and histological remission[74].

Specific antibodies against IL-5 and IgE were also tested, especially for treating steroid-refractory patients. However, despite the initial promising results, the controlled clinical trials have shown a similar response in treated and placebo subgroups[75-78]. Interestingly, a recent trial testing a human antibody against IL-13 showed a significant improvement of symptoms and eosinophilic infiltration, however the primary endpoint, the reduction of 75% of esophageal eosinophils, have been not achieved[79].

The failure of such specific therapy likely depends on the redundancy, in fact multiple cytokines share similar effects, allowing the persistence of inflammation after the blockage of single molecules.

Endoscopic treatment

Due to the fibro-stenotic evolution, strictures are frequently found in patients affected by EoE, hence endoscopic dilation have a key role in the treatment of this entity[1]. Several studies have shown the efficacy and safety of esophageal dilation, independently on the chosen technique. Wire-guided bougies, through-the-scope balloons, and non-wire-guided bougies have been indistinctly used[80,81]. Nowadays, there are not available data on the best endoscopic technique, because comparing studies are lacking.

Due to the long-term efficacy of this procedure in the treatment of EoE-related dysphagia, some authors have proposed dilation as initial therapy, however a recent study have demonstrated that fluticasone inhaler, followed by dilation if necessary, is the most economical initial strategy. Moreover, although dilation rapidly improve symptoms, this procedure did not influenced

esophageal inflammation, hence further therapies should be set after the treatment[82].

CONCLUSION

EoE represents a multifactorial disease, in which both genetic predisposition and environmental factors contribute to disease manifestations. Although in the last years many studies have been performed, its pathophysiology remains unclear, likely reflecting the heterogeneity of disease phenotype.

The EoE-symptoms pattern is heterogeneous, dysphagia and food impaction are frequently referred, however also atypical GERD symptoms may be reported. The histological identification of a prevalent eosinophilic esophageal infiltrate represents the major diagnostic criterion for EoE, however only the PPI-trial allows to distingue EoE from PPI-REE.

The good response to diet therapy in children, supports the role of food as a major trigger factor, leading to define EoE a subtype of food-allergy. For this reason, elimination diets and corticosteroids represent the mainstay of EoE-therapy, while endoscopic dilation have a key role in the treatment of fibrotic complication.

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REVIEW

Host-microbiome interaction in Crohn's disease: A familiar or familial issue?

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Abstract

An impaired interaction between the gut and the intestinal microbiome is likely to be the key element in the pathogenesis of Crohn's disease (CD). Family studies have provided invaluable information on CD pathogenesis and on its etiology. Relatives share the same genetic risk of developing the disease as affected subjects. Relatives also exhibit similar features relating to their host-microbiome interaction, namely genetic variants in loci involved in detecting bacteria, a greater sero-reactivity to microbial components, and an impaired intestinal permeability. The burden of environmental factors such as cigarette smoking and dysbiosis also seems to be particularly relevant in these genetically predisposed subjects. Diet is emerging as an important factor and could account for the changing epidemiology of CD in recent years. Despite the pivotal role of genetics in the disease's pathogenesis (especially in familial CD), screening tests in healthy relatives cannot be recommended.

Key words: Crohn's disease; Genetics; Environment; Microbiome; Relatives

Core tip: Family studies support a host-microbiome interaction in the development of Crohn's disease (CD). Unaffected relatives reveal genetic variants in loci involved in detecting bacteria, a greater sero-reactivity to microbial components, an impaired intestinal permeability, and a greater susceptibility to environmental factors. Whether genetic or environmental factors drive these conditions is still under investigation, but CD pathogenesis is very likely multifactorial. A genetic burden may be hypothesized in familial CD, while environmental factors may be predominant in sporadic CD.

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INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) remains unclear, but is likely to be multifactorial and driven by an aberrant immune response to the gut microbiome in genetically susceptible hosts[1]. The genetic hypothesis to explain the pathogenesis of IBD, and Crohn's disease (CD) in particular, has been intriguing researchers ever since familial clustering was first described by Crohn *et al*[2] himself in the 1930s, but the identification of the genes potentially involved has been hampered by the lack of a classical Mendelian inheritance. The rate of concordance in monozygotic twins is relatively low[3]. There is also a growing body of evidence to support an environmental burden in IBD epidemiology, now that the incidence of IBD is increasing in developing countries such as Asia and Africa[4], in migrants in Western countries[5], and in patients' spouses[6].

Family studies have generated invaluable data regarding the pathogenesis of CD, as relatives may share both genetic and environmental factors with patients. These studies have shed light on the role of host-microbiome interaction in the disease's development. The gut microbiome is involved in general homeostasis, with a crucial role in nutrition, energy metabolism and host defense[7]. The relationship between human organisms and their gut microbiome is regulated by the intestinal mucosal barrier, the permeability of which is a functional property that enables coexistence with bacterial symbionts, while preventing penetration by luminal macromolecules and pathogens[8-11]. Changes in the gut microbiome and intestinal permeability lead to an abnormal mucosal immune system response, which is the final step in the pathogenesis of IBD[12-15]. Both innate and acquired gut immunity participate in maintening a state of chronic inflammation, with activated dendritic cells and mucosal T $CD4^+$ cells apparently playing a key part in antigen presentation and response to the gut microbiome[16,17].

Changes in host-microbiome interactions have been well-documented in both CD patients and their unaffected relatives.

FAMILIAL RISK IN CD

Epidemiological studies have shown that almost 30% of IBD patients have a positive family history of the disease[18-21], which is the most important risk factor for the onset of IBD; the lifetime risk for first-degree relatives of CD patients is as high as 7.8% among Jewish people[20,22-25]. It has been demonstrated that having a sibling with CD coincides with a 30-fold increase in the odds of developing the same illness[16,22], or the other major form of IBD, ulcerative colitis (UC), and viceversa[20,26,27]. This cross-disease effect supports the evidence for a common genetic background in the onset of the two forms of IBD. There is also a cumulative effect since the risk increases when more than one relative or both parents are affected[6,28,29]. Familial CD seems to be a distinct entity from sporadic cases of the disease because it becomes manifest at a younger age and has a different phenotypic expression, with a higher prevalence of ileal involvement[23,29-32], a complicated course with penetrating or extraintestinal manifestations[33], and a strong concordance in terms of the site of disease and its behavior[21,33]. Although not all subsequent studies matched these results[34,35], a recent prospective study on more than six thousand CD patients confirmed them[36]. In families with CD, the children affected also have an earlier age of onset and a more aggressive course of the disease than their affected parents. Some authors suggested that genetic anticipation might explain this picture[37], but genetic anticipation is usually associated with monogenic diseases, and several further studies reveal potential biases due to a greater awareness of the condition[38-41], or generated contradictory results[21,42-44]. Irrespective of family history, pediatric-onset CD has a more aggressive behavior, a higher rate of resistance to therapy, and a particular phenotype and genetic susceptibility[45-47]; hence the Paris pediatric modification of the classical Montreal classification of IBD, which takes the influence of a very early onset on the disease's history into account[48].

Twin studies on the concordance in the disease's development and phenotype have identified a genetic predisposition that is stronger for CD than for UC[45,49,50]. The concordance rate ranges from only 30% to 50% in monozygotic twins, however, meaning that environmental factors cannot be overlooked, as discussed below.

FAMILY STUDIES ON *NOD2*/*CARD15* AND OTHER GENES

The nucleotide oligomerization domain 2 (*NOD2*) gene, later termed caspase recruitment domain 15 (CARD15), was the first to be identified as CD-susceptible in 2001[51,52]. Since then, more than a hundred polymorphisms and mutations have been reported in this gene, but only three of them are independently associated with CD, namely alleles *R720W*, *G908R* and *L1007finsC*[53,54]. Alone, these alleles each confer a risk of CD development that ranges from 1.5 to 3 fold, which rises to more than 40 when there are two of them in homozygosis or compound heterozygosis[55]. NOD2/CARD15 is a putative intracellular pattern recognition receptor expressed in several cells (monocytes, macrophages, intestinal epithelial and Paneth cells) and, when mutated, its ability to detect bacteria by recognizing peptidoglycan is impaired[53,56,57].

NOD2/CARD15 probably has a broader range of action in host-microbiome interactions, however, because its genotype affects gut microbiota composition[58], and its mutations have also been associated with defensin deficiency and an increased mucosal permeability in CD patients[59-62].

NOD2/*CARD15* mutations have been seen equally

often in patients with sporadic and familial CD[63-65], with the exception of one report of a higher frequency in the latter[66]. No differences have been found between relatives from multiplex and simplex families either[67], while they carry mutations significantly more frequently than in the general population[63,68-70].

An Italian multicentric study found that, irrespective of family status, CD patients carrying at least one NOD2/CARD15 variant had a clinically aggressive disease that had been diagnosed at a younger age; they featured ileal involvement, a stenosing pattern and a history of surgical resections[54].

It is worth noting that the prevalence of *NOD2/CARD15* mutations in CD patients is less than 50%, while it reaches 20% in healthy controls. This goes to show that, though important, it explains only a minor part of the variance in the development of CD[53]. A recent meta-analysis confirmed that NOD2/CARD15 mutations have little power in predicting the course of the disease[71].

The hypothesis of a genetic predisposition in the onset of CD is nonetheless consistent with the previous-mentioned family studies, and with epidemiological evidence of ethnic differences[72,73]. In recent years, population-based genome-wide association studies (GWAS), and subsequent GWAS meta-analyses have also led to the detection of more than 160 IBD-associated loci, with more than 30 loci related to CD, and nearly 300 potential candidate genes[3,4,45,74].

These genetic studies have underscored the importance of host-microbiome interactions, highlighting the role of genes involved in barrier function, T-cell subsets, cytokine signaling, autophagy and mycobacteria recognition[74-76]. These novel genetic markers have not been studied as extensively as NOD2/CARD15, but current data do not support any familial association[77-80]. On the other hand, a large international multicentric study on IBD4 (a CD-related locus containing several candidate genes) identified a greater genetic concordance in CD families where at least one member smoked than in non-smoking CD families[81]. This important finding again suggests that the expression of CD in a given patient is the result of interaction not only between the gene products of several susceptibility loci, but also between these products and certain environmental factors.

Currently known variants can predict less than 14% of the IBD risk and they are quite common in the general population too, and associated with other inflammatory diseases[82]. In fact, the limited sensitivity and specificity of these mutations make a genetic screening for relatives unfeasible.

FAMILY STUDIES ON SEROLOGICAL MARKERS

A hyper-responsive adaptive immunological response to microbial antigens is characteristic of CD and several antibodies have already been correlated with this condition, including: Anti-glycans (ASCA directed against mannan of *Saccharomyces cervisiae*, ACCA against chitobioside, ALCA against laminaribioside, AMCA against mannobioside, anti-L against laminarin, and anti-C against chitin), anti-bacterial sequence I2 of *Pseudomonas fluorescens* (anti-I2), anti-bacterial flagellins (CBir, A4-Fla2, Fla-X) and anti-outer membrane porin C of *Escherichia coli* (OMPc)[83,84]. Their clinical utility lies in their non-invasiveness, their ability to differentiate IBD phenotypes, and their prognostic value in CD. No current guidelines recommend their routine detection, however, given their low sensitivity, even though recent works have underscored their diagnostic and prognostic value when used in combination[84,85].

Family studies have demonstrated that some of these antibodies are more expressed in unaffected first-degree relatives of CD patients than in the general population, with a prevalence that reaches 20%-25% for ASCA, 15%-19% for anti-OmpC, 62% for ACCA, and 89% for ALCA[83,86-88]. Using a quantitative detection assay, we also found that serum levels of ACCA, ALCA and AMCA were similar in first-degree relatives and CD patients, and significantly higher than in healthy controls[83]. When we tested the magnitude of the total serological response to microbial antigens (the four anti-glycans and anti-OmpC), we found that first-degree relatives had a weaker response than patients, but a stronger response than healthy controls. Being uninfluenced by household conditions, these results support the hypothesis that CD are genetically predisposed to the development of antibodies against microbiota[83]. These antibodies cannot be an epiphenomenon of immune activation because they are not associated with abnormal intestinal permeability or active disease[85,89]. On the other hand, a genetic background as a predisposing factor for sero-reactivity has emerged from studies on ethnicity[90], and on the heritability of ASCA positivity in twins[91] and multiplex families[67], and from works correlating NOD2/CARD15 with serological markers. Several authors have reported that the aforementioned NOD2/CARD15 polymorphisms predispose individuals to the development of anti-microbial antibodies development[54,92-94], and one study even demonstrated that both CD patients and their unaffected relatives carrying any of these genetic variants, had a higher number of positive antibodies and increased serological semi-quantitative levels[95].

The association between serological response and genetics is likely to be more complex, however, and influenced by other factors, as shown by Vasseur *et al*[67], who reported that the ASCA trait in multiplex families is due partly to CD itself, not just to the *NOD2/CARD15* genotype. Two complementary reports have also shown that, while CD patients with a positive family history have a higher prevalence of antibody and serologic responses[96,97], each additional positive antibody increases the risk of CD, whatever the *NOD2/CARD15* genotype[98].

Since a study suggested that ASCA could predict the onset of IBD[99], there has been increasing interest in the sero-reactivity of IBD patients' relatives. No longitudinal studies on serological markers conducted to date have demonstrated which relatives will develop IBD, however[33]. Although antibody response may vary over time, the risk is probably higher the greater the intensity of the response[98], so quantitative tests on a number of antibodies might be helpful for stratifying the risk of disease in relatives.

FAMILY STUDIES ON INTESTINAL PERMEABILITY

An altered mucosal barrier function and greater intestinal permeability contribute to chronic inflammation in IBD, facilitating the interaction between the enteric immune system and the gut microbiome[13,14].

Several changes have been reported in the components of CD patients' mucosal barrier, mainly involving the intercellular adhesion molecules[100,101]. These changes increase paracellular permeability, nearly by as much as 50% when assessed with sugar excretion tests[102].

A greater paracellular permeability may not just a consequence of mucosal inflammation. It can be seen in IBD patients with quiescent disease too, and it correlates with intestinal symptoms even in the absence of any endoscopic disease activity[103].

The hypothesis of a genetic predisposition to barrier impairment in CD is suggested by the association between genes involved in intestinal barrier homeostasis and IBD susceptibility[104], and supported by the observation that up to 40% of relatives have an altered small intestinal permeability[33,102,105]. We found permeability abnormal in both patients and their relatives, with a more frequent occurrence in familial than sporadic cases of CD, and an association with NOD2/CARD15 variants in multiplex patients[61]. Other authors found not such correlation between permeability and genetic polymorphisms[106-108], but such studies mainly involved sporadic cases of CD.

The role of genetics has also been questioned in the light of an increased permeability being observed in spouses of CD patients[33], and after a recent study underscored the importance of age and environmental factors such as age and smoking, rather than genotype, as contributors to permeability in relatives[109]. Oddly enough, relatives who smoked did not seem to have an altered permeability in this latter study. This is a matter that will need further investigation, however, because smoking is a known risk factor for CD and has recently been associated with a greater permeability of the small intestine in experimental models[110].

In conclusion, the abnormal intestinal permeability found in CD patients' relatives further confirms a link between genetics and environmental factors in the development of CD. Thus far, there has been one only reported case of CD occurring in a relative as predicted by an abnormal permeability test[111], so there is still too little evidence to warrant intestinal permeability assessments in relatives for screening purposes.

FAMILY STUDIES ON ENVIRONMENTAL FACTORS

Some environmental factors shared by family members may contribute to modulating the microbiome and its interaction with the gut immune system. CD patients have a particular dysbiosis involving a reduction in *Clostridium* and *Bacteroides* species[12]. Given the symbiotic relationship between the gut microbiome and the mucosal barrier's integrity, this dysbiosis may aggravate any intestinal permeability impairment. In fact, the bacterial strains that diminish in CD are also the main producers of butyrate, which is fundamental to intestinal cell homeostasis and mucosal barrier integrity[12]. Several efforts have been made to manipulate the gut microbiome in order to restore homeostasis: probiotics, prebiotics and fecal transplantation have generated promising results but their efficacy is short-lived in CD, probably because other host characteristics affect the balance of the intestinal flora[112,113].

Siblings have the same dysbiotic features as CD patients, particularly involving a reduction in *Faecalibacterium prausnitzii*[114]. This may be genetically determined[115], to some degree at least, but a study performed on twins showed that the gut microbiome was associated more with disease phenotype (ileal *vs* colonic CD) than with genotype[116].

Similarly, childhood exposure to environmental factors influencing the intestinal microbiome, such as gastrointestinal infections, antibiotic use and hospitalization, may override the role of genetics, even in twins[117,118]. A recent longitudinal study identified a declining role of childhood exposure to such factors, whereas smoking and family history of the disease remained the main risk factors[119]. Smoking has proved particularly harmful in familial CD, raising its incidence and reducing the age of onset[120]. Together with its previously-mentioned effect on intestinal permeability, smoking may also affect the intestinal microbiome, leading to dysbiosis[121].

Epidemiologic data underscore the importance of environment-driven pathways: The incidence of CD is rising (and more rapidly than that of UC)[122], the colonic phenotype is becoming more common than ileal CD[123,124], monozygotic twin concordance is declining, and pediatric studies have shown a reduction in familial CD and an increasing multiethnicity of cases[50,122]. Western diet is increasingly seen as a major contributor to the changing epidemiology of CD because numerous dietary factors may affect the microbiome and intestinal permeability, leading to an acquired bacterial clearance defect that would foster subsequent mucosal inflammation[125]. Several studies have identified highly-

Table 1 Characteristics of familial and sporadic Crohn's disease in patients and healthy relatives

Familial CD	Sporadic CD
Patients	
Younger age at presentation	Onset al the classical peak age for IBD
Predominantly ileal involvement	Predominantly colonic involvement
Penetrating/stenosing phenotype	Less frequently complicated
More frequent extraintestinal manifestations	Less frequent extraintestinal manifestations
More frequent NOD2/CARD15 mutations	NOD2/CARD15 mutations < 50% of patients
Higher prevalence of anti-glycan antibodies	NOD2/CARD15 mutations associated with an increased sero-reactivity to microbial antigens
Impaired intestinal permeability associated with NOD2/CARD15 variants	Impaired intestinal permeability in < 50% of patients
Environmental factors: Smoking	Environmental factors: Smoking, diet?
Healthy relatives	
Genetic concordance of IBD4 locus in families with smokers	No reported genetic concordance
ASCA trait	Increased sero-reactivity to microbial antigens, also correlating with NOD2/CARD15 genotype
Abnormal intestinal permeability	Abnormal intestinal permeability in < 40% of relatives

IBD: Inflammatory bowel disease; NOD2/CARD15: Nucleotide oligomerization domain 2/caspase recruitment domain 15; ASCA: Anti-*Saccharomyces* antibodies; CD: Crohn's disease.

refined sugars as a major culprit[120], but a recent study suggested that the current burden of immune-related diseases (including CD) may also be explained by the increasing consumption of other industrial food additives - *via* an impaired intestinal permeability[126]. There are currently no family studies on the consumption of such dietary components (earlier research mainly addressed cereal intake and produced contradictory results[127]). There is therefore not enough evidence as yet to support a causal effect of diet on CD, although a proinflammatory effect may be postulated for certain dietary components[118]. As one of the environmental factors, diet is likely to be a major contributor to the increasing incidence of colonic CD, as suggested by twin studies[117,128].

CONCLUSION

Despite the accumulating evidence emerging from genetic studies, the numerous susceptibility loci identified to date explain only a part of the variance in CD risk. Host-microbiome interaction has a pivotal role in CD pathogenesis, although the factor capable of turning a symbiotic into a pathogenic relationship remains unknown[75].

Family studies have generated the strongest evidence of genetic and environmental factors being complementary contributors to microbially-driven inflammation in CD. Maybe, familial and sporadic CD should be considered as different entities (Table 1): The genetic burden prevails in familial CD, in which the genetic background influences the disease's phenotype and course, whereas environmental factors could be more important in the pathogenesis of sporadic cases[50].

Some degree of subclinical inflammation has been demonstrated in healthy relatives of CD patients[117,129,130], but it does not necessarily develop into clinical disease over time[131,132]. This limits the value of non-invasive screening tests, even though such tests proved effective in detecting CD even before it becomes symptomatic[133,134].

In conclusion, CD patients' relatives should not undergo screening so long as they are symptom-free, but they deserve special attention because of the invaluable information they can provide on the disease's pathogenesis.

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REVIEW

Gastrointestinal dysbiosis and the use of fecal microbial transplantation in *Clostridium difficile* infection

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Abstract

The impact of antibiotics on the human gut microbiota is a significant concern. Antibiotic-associated diarrhea has been on the rise for the past few decades with the increasing usage of antibiotics. *Clostridium difficile* infections (CDI) have become one of the most prominent types of infectious diarrheal disease, with dramatically increased incidence in both the hospital and community setting worldwide. Studies show that variability in the innate host response may in part impact upon CDI severity in patients. That being said, CDI is a disease that shows the most prominent links to alterations to the gut microbiota, in both cause and treatment. With recurrence rates still relatively high, it is important to explore alternative therapies to CDI. Fecal microbiota transplantation (FMT) and other types of bacteriotherapy have become exciting avenues of treatment for CDI. Recent clinical trials have generated excitement for the use of FMT as a therapeutic option for CDI; however, the exact components of the human gut microbiota needed for protection against CDI have remained elusive. Additional investigations on the effects of antibiotics on the human gut microbiota and subsequent CDI will help reduce the socioeconomic burden of CDI and potentially lead to new therapeutic modalities.

Key words: Human gut microbiota; Antibiotic-associated diarrhea; Fecal microbial transplant; Bacteriotherapy; Dysbiosis

Core tip: Emergent literature demonstrates the critical

role of the human microbiota in the susceptibility to *Clostridium difficile* (*C. difficile*) infection (CDI). Microbial communities may exert effects on the metabolic composition within the GI tract that influence CDI pathogenesis (*e.g.*, bile salt metabolism). The identification of protective and susceptible human gut microbiomes would enable the development of screening tools to identify at-risk patients. Ultimately, the rational design of probiotic cocktails could assist in attenuating *C. difficile* transmission in hospital or community settings. Prevention of CDI would lead to decreased morbidity and mortality, as well as reduction of hospitalization time and health care costs associated with treatment.

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INTRODUCTION

The discovery and application of antibiotics in the early 20th century was one of the most influential breakthroughs in medical history. It has led to the treatment of many diseases and improved survival rates of serious wounds and surgeries. However, the antibiotics used to treat these infections are not always specifically targeted towards the disease-causing bacteria, as broad-spectrum antibiotics can target other bacteria, including the commensal bacteria in the human intestinal tract, known as the human gut microbiota (HGM). Furthermore, calls from various health agencies and ministries have been made for improved antibiotic stewardship, as over-prescription of antibiotics has led to escalations in antibiotic-resistant bacteria[1-4], including *Clostridium difficile* (*C. difficile*)[5]. While the increases in antibiotic resistance cannot be over looked, the indirect impact of antibiotics on the HGM is a growing problem. Antibiotic-associated diarrhea (AAD) has been on the rise for the past few decades with the increasing usage of antibiotics. *C. difficile* infection (CDI) has become one of the most prominent types of AAD with increasing rates in both the hospital and community setting worldwide[6-8]. This increased burden on both the patient population and healthcare costs has become an alarming predicament. Investigations into the effects of antibiotics on the HGM and subsequent CDI will help reduce this burden and potentially lead to new therapeutics to treat this emergent epidemic.

THE HUMAN GUT MICROBIOTA

The human gastrointestinal tract houses one of the most dynamic bacterial communities on the planet, with hundreds of species and thousands of strains competing for nutrients while producing by-products that can be beneficial to the host. Additionally, the HGM plays a key role in host defense, providing a protective and competitive layer to resist growth of different pathogens. However, dysbiosis and overgrowth of microbiota has been seen in several different diseases, including inflammatory bowel disease (IBD)[9] and irritable bowel syndrome[10], allergy and asthma[11], tumorigenesis[12], nonalcoholic fatty liver disease[13], cardiovascular disease[14], autism[15,16], and obesity[17,18]. It is clear that proper maintenance and turnover of the microbiota is essential for proper health.

The HGM plays a large role in health and disease, yet until recently much of its composition and function remained unknown[19]. The HGM has been shown to play important roles in early life development, including vitamin and nutrient absorption, stimulation of intestinal angiogenesis, protection from pathogens and immune development[20]. This complex ecosystem is rapidly responding to the harsh environment within the gastrointestinal tract, including nutrient and pH fluctuations, niche competition with other bacteria, and antimicrobial peptides being excreted by the host[21]. While specific bacterial pathogens are well known to cause discrete disease, overall bacterial dysbiosis has been linked to many chronic diseases that are prevalent in society[16]. The mutualism exhibited by the HGM and its human host is paralleled by few other host-microbe interactions. Ultimately, knowledge of the HGM could be utilized to analyze disease status and therapeutic responses.

METHODOLOGY FOR STUDYING THE HUMAN GUT MICROBIOTA

The study of the gut microbiota has been ongoing for almost a century. The earliest studies isolated animals into a germ-free state, allowing development without any influence from bacteria or their products. In answer to a challenge originally issued by Louis Pasteur, scientists at the University of Notre Dame were able to deliver guinea pigs by caesarean section and house them in germ-free containment. These guinea pigs were then bred under germ-free conditions, to provide axenic animals[22]. The hope was for the animals to be used in identifying bacterial roles in normal physiology, as well as their role in proper immune defense against bacteria, as antibiotic resistance was already a rising threat.

Bacteria from the HGM are difficult to isolate in culture, making it challenging to determine the overall microbial community. The study of the HGM has expanded in the last decade due to advances in culture-independent methods of monitoring microbial diversity. Most techniques target the 16S ribosomal RNA (*16S rRNA*) gene, as it contains conserved and variable regions that can be used to distinguish different bacterial species[23]. The *16S rRNA* gene is approximately 1500

nucleotide base pairs in length, and contains nine conserved regions and nine variable regions (V1-V9)[23]. These variable regions have been targeted for bacterial group and species identification *via* different techniques. Basic techniques that began the culture-independent revolution include denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP).

Introduced in 1993, DGGE provided a snapshot of overall microbial diversity within a sample. The *16S rRNA* gene is amplified in a polymerase chain reaction (PCR) and subjected to separation in polyacrylamide containing a denaturing gradient of urea and formamide[24]. This allowed DNA fragments to be separated based on melting temperature, fragment length and guanine-cytosine content, resulting in a unique band pattern. These band patterns can be compared to prepared bacterial standards, or bands can be excised and analyzed by sequencing[24]. However, multiple bacterial taxa could occupy one band in a gel such that underestimation of bacterial diversity frequently occurred[25].

TRFLP, first described in 1997, involves PCR amplification of the *16S rRNA* gene using a fluorescently-labelled forward primer[26]. This reaction is followed by restriction digestion targeted towards a conserved sequence, which produces terminal-restriction fragments (TRFs). Fluorescent primers allow for quantification of the TRFs, giving an overall and relative abundance. Identification to the phyla or deeper levels was possible by comparing the TRFs to an *in silico* library, which was generated from the Ribosomal Database project[27].

The strengths of DGGE and TRFLP in the early 2000's included relatively high throughput combined with lower costs in an age of expensive genomic sequencing technology. Both methods were able to provide a fingerprint of the microbial community within a fecal sample and enabled inter-sample comparisons. However, both were also associated with relatively poor resolution of the HGM community members, sometimes failing to identify bacteria beyond the phyla level. With the development of new and more cost-effective genomic sequencing [*i.e.*, next-generation sequencing (NGS) methods[28]], the method is now at the forefront of HGM research. While sequencing of the HGM has become for readily available, the bioinformatic analysis still provides challenges for the facile interpretation of datasets. Several computing programs and pipelines have been assembled, primarily through the Human Microbiome Project, which aid in the analysis[29]. "Quantitative insights into microbial ecology", or QIIME, has been the most prominent pipeline developed thus far, as it allows the input of thousands of sequences and navigates through sequence alignment to known databases and downstream community analysis[30]. With the development of rapid and feasible sequencing, as well as improved bioinformatic output, the study of the HGM has shifted to culture-independent dominated processes. However, the cultivation of bacteria is still considered the gold standard, and microbial DNA in samples does not indicate whether the sample came from an active or dead bacteria[31]. Nonetheless, the NGS technologies have allowed for new studies to examine the HGM in health and disease.

C. difficile

C. difficile is an anaerobic, spore-forming bacterium that was originally identified as a natural part of the infant microbiota[32]. However, research since the 1970's has linked *C. difficile* colonization as the primary cause of antibiotic-associated and nosocomial diarrhea in the adult and elderly populations[33,34]. CDI symptoms vary amongst patients, ranging from mild to severe diarrhea (> 15 bowel movements per day), with severe cases resulting in toxic megacolon or death[35]. The CDI mortality rate has been increasing over the last decade, due to the development of hypervirulent and antibiotic-resistant strains[36,37]. *C. difficile* transmission has become a major problem in hospitals across the developed world, as *C. difficile* spores are highly resistant to normal cleaning agents, including alcohol-based hand washes.

EPIDEMIOLOGY AND RISK FACTORS FOR CDI

C. difficile has risen to prominence internationally, with several large breakouts within hospitals causing the death of many patients. In 2003, an outbreak of the hypervirulent *C. difficile*, strain North American pulsed-field gel electrophoresis type-1 (NAP1), in Quebec hospitals caused the death of nearly 2000 people[38]. Recently, it was also demonstrated that the spread of the NAP1 strain worldwide originated from this Quebec breakout[37]. In the United States and Europe, hospital-associated CDI is estimated to incur annual healthcare costs over $4 billion[36]. Furthermore, there is increasing incidence in developing countries in Asia, although not from the NAP1 strain[7]. There are several risk factors with high association with CDI, including recent antibiotic exposure, age (> 65 years), recent hospitalization, and proton pump inhibitor use[35]. Antibiotic exposure shows the highest correlation, with odds ratios ranging from 1.31-1.87[39]. Certain antibiotics also demonstrate higher correlations, such as clindamycin[39]; indeed, this finding correlates with the history of the disease since CDI was originally referred to as clindamycin-associated pseudomembranous colitis[33]. There is also an increased threat from community-acquired CDI (CA-CDI), which is less studied compared to hospital-acquired CDI (HA-CDI), and the corresponding incidents of CA-CDI have increased approximately 5-fold over the last decade as well[40]. CA-CDI is normally defined as an outpatient presenting with *C. difficile* toxin-positive stool, or an inpatient presenting less than 2 d after admission. A recent study suggested that the risk factors in these patients were different compared to HA-CDI, as 36% of patients did not have recent antibiotic exposure[41]. Another investigation determined that advanced age is also not a factor, as CA-CDI patients were significantly

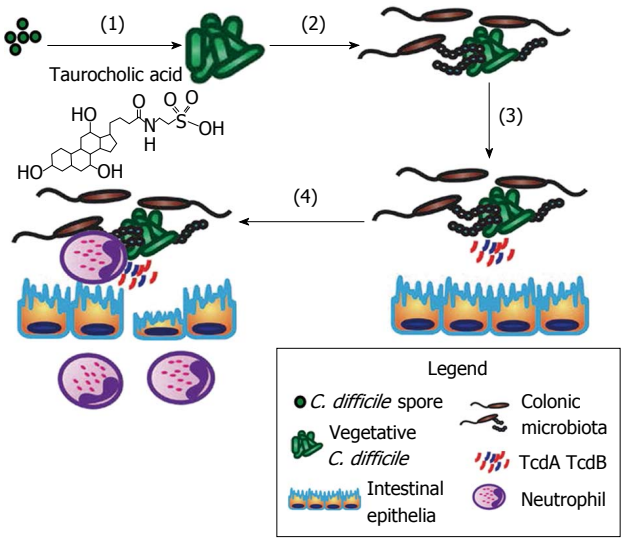

Figure 1 Pathogenesis of *Clostridium difficile* infections. *Clostridium difficile* (*C. difficile*) spores are ingested and pass through the stomach, upon which they can interact with taurocholate within the small intestine and germinate into vegetative cells (1); Vegetative cells then colonize within the gut microbiota (2) and begin to produce toxins, TcdA and TcdB (3); Toxins cause epithelial damage and pro-inflammatory cytokine release, leading to infiltration of neutrophils that cause pseudomembranous colitis (4).

younger compared to HA-CDI[40]. An interesting area of study is the source of *C. difficile* within the community, as hospitals are the normal "reservoir" Recent studies have revealed relatively common contamination of retail prepared foods, including meat, seafood and vegetables, with *C. difficile* spores[42-45]. While CA-CDI risk factors and sources differ from HA-CDI, the increasing rates in both are alarming.

C. DIFFICILE PATHOGENESIS AND LIFECYCLE

C. difficile, as a pathogen, has an interesting lifecycle that ensures its success. Although anaerobic, *C. difficile* can survive for months on aerobic surfaces (*e.g.*, hospital walls, doors, surgical tools, cell phones, *etc.*) in spore form. The spore structure contains several layers, including an exosporium, coat, cortex, membrane, and a DNA-containing core[46]. This makes the spores resistant to alcohol-based cleaning agents used commonly in hospitals. *C. difficile* pathogenesis involves germination of spores into vegetative cells, colonization within the gut microbiota, productions of toxins which lead to toxin-induced intestinal damage and inflammation (Figure 1). When ingested, the multiple layers of the spore help protect it from stomach acids and digestive enzymes. Spore germination occurs upon interaction with the appropriate germinants within the intestinal tract, which include taurocholic acid, a taurine-conjugated bile acid, and glycine[47,48]. The receptor for taurocholic acid on a *C. difficile* spore, CspC, was only recently discovered[49], and researchers demonstrated that mutations to this protein changed germination dynamics. Indeed, mutated CspC led to decreased mortality in the hamster model of CDI. Germination results in the upregulation of several genes, and the entry into the vegetative state of the *C. difficile* lifecycle. This involves the breakdown of the spore cortex, core expansion and water uptake into the core, allowing an increase in enzymatic activity[50]. This complex process has not been well studied in *C. difficile* but has in related species[51]. Germination normally occurs within the cecum and colon as other factors in the small intestine, such as high concentration of chenodeoxycholic acid, act to suppress wide-scale germination[48].

Subsequently, *C. difficile* initiates the activation of the pathogenicity locus (PaLoc) in the genome. The PaLoc, approximately 19.6 kb in size, is composed of 5 genes, *tcdA, tcdB, tcdC, tcdR*, and *tcdE*, which are responsible for the production of two large clostridial toxins, A (TcdA) and B (TcdB)[52,53]. The gene *tcdR*, which is found upstream of the toxin genes, is a positive regulator of gene expression whereas *tcdC* is a negative regulator. A pore-forming holin is encoded by *tcdE*, which allows the release of TcdA and TcdB. Literature has suggested that hypervirulent NAP1 *C. difficile* released more toxin due to decreased expression of *tcdC*; however, this has come into question due to recent evidence that demonstrates no change in toxin production[54]. Another gene regulator is CodY, which binds and represses *tcdR* and thus inhibits toxin production, when essential nutrients are not available[55].

Importantly, CDI-induced colitis only occurs when either of TcdA or TcdB is present[56]. Indeed, patients with pseudomembranous colitis are *C. difficile* toxin-positive > 96% of the time[57]. TcdA was originally believed to be the only toxin necessary for virulence until the discovery of $TcdA^-/TcdB^+$ *C. difficile* strain in major breakouts worldwide[58]; however, $TcdA^+/TcdB^-$ strains are equally likely to cause disease[59]. Recent studies in animals have attempted to delineate the importance of each toxin *in vivo*; new animal model to study host response to intrarectal instillation of *C. difficile* toxins revealed that TcdA was important for the majority of the damage, whereas TcdB alone caused no damage to the mouse colon but could potentiate the effects of TcdA[60]. Interestingly, individuals infected with the same *C. difficile* strain can respond very differently. While the mechanism has not been elucidated, it has been linked to development of antibodies against TcdA and/or TcdB[61,62]. However, this theory has come into question due to the increasing recurrence rates, with recent literature demonstrating that asymptomatic carriers and diseased patients having similar antibody loads towards the toxins[63]. Differences in microbiota between patients with a single case *vs* recurrent CDI have been suggested to play a role in patient susceptibility and variability[64]. Different animal models have been employed to determine important bacterial and host factors involved in CDI.

THE HOST IMMUNE RESPONSE TO *C. DIFFICILE* INFECTION

C. difficile toxins induce epithelial injury, barrier dysfunction and activation of the mucosal immune system[52]. The classic endoscopic and histological feature of CDI is pseudo-membranous colitis, characterized by severe inflammation (neutrophilic/monocytic), ulceration and pseudomembranes[33]. CDI has a rapid onset and previous exposure does not confer protection. There is marked variability in disease severity in patients with similar risk factors who are infected with the same *C. difficile* strain. Some of the variability in responses might, in part, be due to altered innate immune pathways. For example, the nucleotide-binding domain leucine-rich repeat family of genes contains a number of intracellular innate immune receptors that respond to a variety of microbial and non-microbial danger signals. Indeed, TcdA and TcdB can trigger pro-inflammatory interleukin-1B release by activating an intracellular inflammasome[65]. A number of studies also support the activation of humoral immune responses to *C. difficile* toxin proteins during CDI (reviewed in[66]). Most healthy adults have detectable serum antibody titers to TcdA and TcdB (as well as non-toxin antigens) that may originate from transient environmental exposure to *C. difficile* from infancy[67]. The anti-toxin antibody responses (*e.g.*, IgG) in relation to the clinical course of CDI, as well as disease recurrence, have been reported in a number of studies[62,68-71]. Immunity to TcdB may be important in the early stages of CDI; however, antigenic variation in TcdB suggests that acquired immunity may not provide cross-protection among different *C. difficile* strains[62]. Researchers are now examining the efficacy of protective immunity provided by vaccines against *C. difficile*.

TREATMENT OF *C. DIFFICILE* INFECTION

The current therapeutic paradigm for CDI is the removal of the causative antibiotics (*i.e.*, clindamycin) and treatment with vancomycin (complicated disease) or metronidazole (mild disease)[72]. Fidaxomicin is a therapeutic option for patients with recurrent CDI or a high risk of recurrence. Occasionally, when CDI progresses to toxic megacolon, colectomy is required. A recent study demonstrated that survival rates post-colectomy are low, identifying this as a last-resort in CDI treatment[73]. Oral metronidazole was the normal first-line treatment, where vancomycin is used in more severe cases or with metronidazole failure[74]. These guidelines were confirmed by a double-blind, randomized, placebo-controlled clinical trial, showing equal efficacy with vancomycin being more successful in severe patients[75]. However, recurrence of CDI, caused by relapse or re-infection, occurs in about 20%-35% of these patients[76]. Fidaxomicin is a recently introduced antibiotic that targets *C. difficile* more selectively and shows equal efficacy compared to vancomycin[77,78]. This is likely due to the fact that fidaxomicin has reduced effects on the commensal gut microbiota compared to vancomycin. Importantly, there was also decreased CDI recurrence rates in patients taking fidaxomicin compared to vancomycin, highlighting its role as a superior alternative antibiotic[77,78]. However, with recurrence rates still relatively high, it is important to explore alternative therapies to CDI. In this regard, fecal microbiota transplantation (FMT) and other types of bacteriotherapy have become exciting avenues for the treatment for CDI.

COLONIZATION RESISTANCE AGAINST *C. DIFFICILE*

Identifying mechanisms by which the microbiota controls colonization by *C. difficile* and susceptibility to CDI, known as colonization resistance, has been pushed to the forefront of research[79-83]. *C. difficile* pathogenesis requires spore germination, colonization, and toxin production, which lead to the host immune response. Hypotheses for colonization resistance mechanisms include inhibiting germination, limiting important nutrients for colonization, or stimulation of the host immune response. Germination of *C. difficile* spores into vegetative, toxin-producing cells can be inhibited *via* several pathways. As previously mentioned, taurocholate is a primary bile salt that is formed in the liver and is involved in initiating the germination of *C. difficile* spores[84]. There are a few mechanisms by which bacteria actively modify the structure of bile salts, rendering them unable to stimulate germination. Some bacteria produce bile salt hydrolases (BSH) that catalyze the deconjugation of the amino acid (taurine or glycine) from carbon-24 (C-24) of bile salts[85]. BSHs tend to be intracellular enzymes, though one species, *Clostridium perfringens*, produces extracellular BSH[86]. Bile hydrolysis is quite common for different gut bacteria and has been demonstrated in *Clostridia*, *Bacteroides*, *Parabacteroides*, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*[85,87-89]. BSHs are hypothesized to be beneficial to commensal bacteria by liberating nutrients (*e.g.*, amino acids[90]), possibly giving a competitive advantage to BSH-producing bacteria. Additional studies have demonstrated that increasing *BSH* gene copies in Listeria result in increased survival *in vivo*, while decreasing BSH expression results in decreased bacterial growth[91].

Specific members of *Eubacterium* and *Clostridia* genera have the ability to epimerize bile acids at the C-7 position (conversion of 7α- to 7β-hydroxy), converting primary bile salts (cholate and chenodeoxycholate) into secondary bile acids (deoxycholic acid and lithocholic acid, respectively) *via* 7α-dehydroxysteroid dehydrogenases (7α-HSDH) and 7β-HSDH[92]. These enzymes exist solely in the large intestine of humans[85]. HSDHs also exist for the C-3 and C-12 positions on bile salts. Bacteria also produce dehydroxylation enzymes, which are important in the excretion of bile salts. Interestingly, these enzymes

can only function on deconjugated bile acids, requiring the activity of BSHs first[93]. This requires the bacteria to either produce BSH, or live in close proximity to a microbe that does. Taurocholate is rarely available to allow *C. difficile* to germinate in a healthy gut given the constant catabolism of bile salts. However, recent studies have identified that exposure to antibiotics decreases the metabolism rate of primary bile salts. Naïve mouse cecal contents incubated with *C. difficile* spores are unable to permit germination of *C. difficile in vitro*[47]. However, intraperitoneal injections of clindamycin and subsequent cecal content incubation led to *C. difficile* germination. This was corroborated with increased primary bile salts and decreased secondary bile salts in the cecum[47]. Additionally, incubating taurocholate with isolated cecal microbiota from naïve mice resulted in breakdown of the majority of the primary bile salt, whereas clindamycin-treated mice completely lost their ability to metabolize taurocholate. This was recently corroborated with a metabolomic study that assessed bile in cecal contents of mice[94]. Most recently, analyses of the HGM of hospitalized CDI patients identified resistance-associated bacteria[80]. *Clostridium scindens* (*C. scindens*), a bile acid 7α-dehydroxylating intestinal bacterium, was associated with colonization resistance. Probiotic administration of *C. scindens* provided resistance to CDI in a secondary bile acid dependent fashion. Taken together, these studies demonstrate a potential link between microbiota function, bile metabolism and CDI susceptibility.

Pharmacologic agents that target the interaction between *C. difficile* spores and taurocholate have also been investigated as a potential therapeutic option[95]. A bile salt analog, cholate meta-benzene sulfonic acid (CamSA), was a strong inhibitor of *C. difficile* germination *in vitro* and *in vivo*. In this case, CamSA (50 mg/kg) was able to completely inhibit *C. difficile* germination in a mouse, resulting in no CDI pathology[95]. Interestingly, a bile salt sequestrant, cholestyramine, was previously used as an adjunct therapy with antibiotics for CDI[96]. The mechanism of action was considered to be binding of *C. difficile* toxins but could have also been associated with *C. difficile* spore germination. One case study also detailed a patient with recurring CDI who was cured after prolonged cholestyramine therapy, potentially due to decreased germination[97]. Clearly, alteration of *C. difficile* germination is able to play a protective role in CDI.

ANTAGONISTIC ACTIVITY OF COMMENSAL BACTERIA AGAINST *C. DIFFICILE*

In recent years, the aim for developing treatment for CDI has been a narrow-spectrum antibiotic against *C. difficile* (*e.g.*, fidaxomicin) and microbiota sparing[75-78]. However, treatment strategies that rely on antibiotics impose strong selection for resistance as well as the disruption of the normal microbiota. Members of the gut microbiota can also produce antimicrobial compounds, termed bacteriocins, which target a narrow range of bacterial species. Researchers in Ireland identified a *C. difficile*-targeting bacteriocin, Thuricin CD, produced by *Bacillus thuringensis*[98]. Thuricin CD was shown to be as effective as antibiotics *in vitro* for the elimination of *C. difficile*, while also having limited impact on the host microbiota. The group recently published that intrarectal instillation of Thuricin CD into mice was able to reduce shed *C. difficile* in the feces, though showed low bioavailability when orally gavaged[99]. Another contractile bacteriocin protein complex (R-type; diffocin) was engineered to kill specific *C. difficile* pathogens[81]. The diffocins (*i.e.*, Avidocin-CDs) prevented colonization of NAP1-type *C. difficile* strains and limited their transmission. Avidocin-CDs administered in drinking water survived passage through the mouse gastrointestinal tract, did not detectably alter the mouse intestinal microbiota and did not disrupt natural colonization resistance to *C. difficile*.

A group at the University of Michigan identified that antibiotic-treated mice had reduced levels of *Lachnospiraceae* in their feces, which correlated with increased CDI severity[100]. Reeves *et al*[101] later demonstrated that, while germ-free mice were extremely susceptible to CDI, the addition of multiple Lachnospiraceae family members suppressed the growth of *C. difficile* by 20-fold, and decreased the toxin production by 25%. Only complete cecal microbiota transfer entirely inhibited CDI. This parallels with studies completed in the 1980's, where Itoh *et al*[102] transferred several different species, but only a full fecal transfer conferred protection.

Different groups have looked at the ability of certain bacterial species to inhibit toxin production *in vitro*. Kolling *et al*[103] determined that *Streptococcus thermophilus* produced lactic acid, which was able to suppress the transcription of tcdA and release of TcdA *in vitro*. Furthermore, mice treated with *Streptococcus thermophilus* have less severe CDI with decreases in weight loss, tissue injury on pathology, diarrhea, detectable *C. difficile* bacteria and toxin levels. This study demonstrated the ability of bacteria and bacterial products to suppress *C. difficile* growth and toxin production.

A recent study by Lawley *et al*[104] identified a consortium of six bacterial species in mice that could treat a recurrent carrier-model of CDI. These species, *Bacteroidetes* sp. nov., *Enterorhabdus* sp. nov., *Enterococcus hirae*, *Lactobacillus reuteri*, *Staphylococcus warneri*, and *Anaerostipes* sp. nov., were able to reduce inflammatory parameters of CDI as well as fecal counts of *C. difficile*. Individually, these six species were unable to confer protection, and no metabolic pathways could be identified to rationalize the effectiveness of the treatment[104]. The addition of these species did lead to increased bacterial diversity within the mouse intestinal microbiota, which was suggested as the potential mechanism for restoring colonization resistance. However, these species are limited to mice and not seen in HGM, so its application to human CDI is not clear.

FMT

FMT has risen to prominence in the past few years as an exciting therapeutic approach for CDI, especially recurrent CDI. However, FMT actually has a very extensive history and has been successfully used to treat diarrhea for over 50 years. Four patients were treated with fecal enemas in 1958 to resolve pseudomembranous colitis, and they exhibited dramatic resolution over 24-48 h post-treatment[105]. This study was conducted before *C. difficile* was recognized as the primary cause of pseudomembranous colitis, but it has since been validated by other studies using fecal enema[106]. The current protocol for involves fairly intense screening followed by simple techniques[107,108]. First, the FMT recipient ceases antibiotics and an FMT donor is selected. A donor completes a questionnaire and is screened for different pathogens, including bacteria (*C. difficile*, *Listeria monocytogenes*, *Vibrio cholera*, *Helicobacter pylori*, *Treponema pallidum*), parasites (Giardia, Cryptosporidium), and viruses (rotavirus, hepatitis A/B/C, Creutzfeldt-Jakob, and human immunodeficiency virus)[109]. Donors can also be excluded if they have had recent antibiotics or tattoos, or a history of gastrointestinal disease. Stool is then collected and prepared for transplant (*e.g.*, diluted and homogenized before filtration through gauze pads to remove large particulate matter), although a recent study has demonstrated that frozen/thawed stool works as well as fresh stool[110,111]. The fecal filtrate can then delivered by oral capsule, nasogastric tube, colonoscopy or rectal enema.

Many trials interrogating the efficacy of FMT for CDI have taken place worldwide over the past few years[112-116]. Two healthy donors were used for 27 patients in a recent trial at McMaster University (Hamilton, Canada), showing an overall cure rate of 93%[112]. In this study, failure of FMT was linked to lack of retention of enema. A smaller study from the University of Toronto (Toronto, Canada) examined the different microbiota profiles involved in successful *vs* failed FMT[113]. The study identified several groups of bacteria (reduction in *Bacteroidetes*, increase in *Proteobacteria*) that were important in CDI cases, but indicated that overall diversity was important for success of FMT. The first controlled trial of nasogastric/duodenal infusion of fecal filtrate was published with encouraging results[114]. The FMT arm demonstrated 81% success (13/16 patients) after a single infusion of donor feces, and two of the three failed patients were later successfully treated after infusion of feces from a different donor, for an overall success rate of 94%. This was significantly better than the 31% success (4/13 patients) for standard vancomycin treatment. In a multicenter retrospective series, the use of FMT was examined in immunocompromised patients with CDI that was recurrent, refractory, or severe[115]. The cure rate after a single FMT was 78%, and 89% after repeat FMT in 99 patients in various states of immunosuppression (*e.g.*, human immunodeficiency virus/acquired immune deficiency syndrome, solid organ transplant, chemotherapy, immunosuppressive therapy for IBD). Some patients (14% of IBD patients) experienced disease flare post FMT. Importantly, there were no related infectious complications reported in these high-risk patients.

A systematic review in 2013 found that different forms of FMT had been used to treat 273 patients with confirmed CDI from 1946-2012[117] with an overall success rate of 89% for FMT in treating CDI. In this analysis, a higher FMT success was correlated with lower gastrointestinal delivery routes (colonoscopy and enema *vs* nasoduodenal), but that donor relationship with recipient was uncorrelated. A more recent systematic review completed in 2015 suggests that FMT was associated with symptom resolution of recurrent CDI but its role in primary and severe CDI was not established[72]. The report concludes that treatment strategies should be aligned with disease severity, history of prior CDI, and the patient's risk of recurrence. The exact mechanism by which FMT works on CDI has remained elusive, though it has been suggested to act through the restoration of colonization resistance. Unfortunately, there are also significant drawbacks of FMT. The screening process for known bacterial and viral pathogens can be costly and accessibility to an appropriate donor in a timely manner may be challenging. Additionally, a recent case report of FMT treatment detailed a patient with CDI that resulted in flare of dormant ulcerative colitis[118]. This adverse event suggests that microbial species that are protective in some disease states may be deleterious in others. Furthermore, links between the HGM and extra-intestinal diseases, including type Ⅱ diabetes, obesity and behavioural disorders, have suggested FMT could have long-term safety issues[119-121]. Therefore, a defined and refined bacterial cocktail would be beneficial for treating CDI and avoiding deleterious effects.

Other bacteriotherapy attempts have been made to replace donor stool as the means of conveyance for HGM. These methods generally involve the development of defined bacterial mixtures from laboratory bioreactors. Tvede and Rask-Madsen demonstrated that a mixture of ten different bacteria could be curative to five (out of five) CDI patients in 1989[122]. The investigators found that recurrent CDI patients had decreased *Bacteroides* spp. that recovered after bacteriotherapy. A more recent study, termed rePOOPulation, used a combination of 33 bacteria to successfully treat two CDI patients[123]. These bacteria were cultured from a stool-sample and grown in the laboratory, but study design allowed for the transfer of a reproducible, known quantity of bacteria. However, these studies have still not identified a key mechanism provided by FMT to cure CDI. Identifying how the microbiota interacts and resists colonization by *C. difficile* will result in rational design of probiotic prevention or treatment of CDI.

CONCLUSION

The excitement of FMT as a treatment for recurrent CDI is increasing. A recent commentary has suggested that FMT could potentially be used to treat all cases

of CDI, including a patient's first case[124]. While this suggestion is exciting, detailed safety profiling is still required before FMT is a widespread therapy[108,125,126]. That being said, CDI is a disease that shows the most prominent links to alterations to the HGM, in both cause and treatment. Studies investigating the effectiveness of FMT for CDI are leading to a better understanding of the roles of the microbial community in both host health and disease. Nonetheless, elucidating a specific combination of bacteria that can treat or prevent future CDI cases would be an impactful discovery for the advancement of bacteriotherapy as a viable treatment.

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REVIEW

Lymphoproliferative disorders in inflammatory bowel disease patients on immunosuppression: Lessons from other inflammatory disorders

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Conflict-of-interest statement: Lam GY has no conflicts of interest to declare. Peters AC has been an advisory board member for Janssen, Roche, Lundbeck and Seattle Genetics. Halloran BP is a consultant and/or advisory board member for Abbvie and Janssen. Fedorak RN is a consultant or advisory board member of Abbvie, Ferring, Janssen, Shire, VSL#3, Celltrion. He is also the recipient of the following clinical/basic science research grants: Abbvie, Alba, Bristol Myers Squibb, Centocor, GSK, Genentec, Janssen, Merck, Millennium, Novartis, Pfizer, Proctor and Gamble, Roche, VSL#3, Celltrion. Finally, he is also the owner/Shareholder of Metablolomic Technologies Inc (www.metabolomictechnologies.ca).

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Abstract

Immunosuppressive agents, such as thiopurines, methotrexate, and biologics, have revolutionized the treatment of inflammatory bowel disease (IBD). However, a number of case reports, case control studies and retrospective studies over the last decade have identified a concerning link between immunosuppression and lymphoproliferative disorders (LPDs), the oncological phenomenon whereby lymphocytes divide uncontrollably. These LPDs have been associated with Epstein-Barr virus (EBV) infection in which the virus provides the impetus for malignant transformation while immunosuppression hampers the immune system's ability to detect and clear these malignant cells. As such, the use of immunosuppressive agents may come at the cost of increased risk of developing LPD. While little is known about the LPD risk in IBD, more is known about immunosuppression in the post-transplantation setting and the development of EBV associated post-transplantation lymphoproliferative disorders (PTLD). In review of the PTLD literature, evidence is available to demonstrate that certain immune suppressants such as cyclosporine and T-lymphocyte modulators in particular are associated with an increased risk of PTLD development. As well, high doses of immunosuppressive agents and multiple immunosuppressive agent use are also linked to increased PTLD development. Here,

we discuss these findings in context of IBD and what future studies can be taken to understand and reduce the risk of EBV-associated LPD development from immunosuppression use in IBD.

Key words: Epstein-Barr virus; Immunosuppression; Post-transplantation lymphoproliferative disorders; Lymphoproliferative disorders; Inflammatory bowel disease

Core tip: Immunosuppressive agents, such as thiopurines, methotrexate, and biologics, have revolutionized the treatment and maintenance therapy of inflammatory bowel disease (IBD). However, their use may come at the cost of increased risk of developing lymphoproliferative disorders (LPD). While little is known about this risk in IBD, more is known about immunosuppression risk in the fields of rheumatoid arthritis and post-transplantation with regards to the development of Epstein-Barr virus (EBV) associated LPD. Here, we attempt to review lymphoma risk in the setting of immunosuppression use in various medical conditions, discuss what lessons may be translatable to the IBD field and what future directions can be taken to reduce the risk of EBV-associated LPD from immunosuppression use in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a term that describes a collection of autoimmune gastrointestinal conditions, most notably Crohn's disease (CD) and ulcerative colitis (UC). While UC is confined to the colon, CD can involve the entire digestive tract from mouth to anus. The pathogenesis of IBD is currently thought to be the result of a combination of host/genetic, environmental and microbial factors that perpetuate chronic and inappropriate inflammation of the gastrointestinal tract[1]. IBD has a bimodal age distribution with first diagnoses occurring between 15 to 40 years of age or 50 to 80 years of age[2]. In addition to age, a range of other risk factors have been linked to the development of IBD including gender, ethnicity, smoking, gut microbiome and medications[3]. One concerning consequence of IBD, and its treatment, is the increased incidence of lymphoproliferative diseases (LPD). LPDs include B- and T-cell lymphoma, the development of which can be the result of Epstein-Barr virus (EBV) mediated malignant transformation of normal B- and T- lymphocytes to divide uncontrollably. Other pathogens, such as other human T-cell lymphotropic virus-1, human herpesvirus-8, hepatitis B and C, human papilloma virus, Kaposi's sarcoma-associated herpesvirus, Merkel cell polyomavirus and *Helicobacter pylori* have also been implicated in malignant transformation of the infected host[4,5]. LPD encompasses a diverse group of hematological malignancies that can either be acute or chronic in nature; either leukemic or lymphoid in morphology. One unique group of LPD includes the post-transplantation lymphoproliferative disorders (PTLD), which can develop due to both primary and secondary immunosuppression[6]. IBD itself, even independent of immunosuppressive treatment, is thought to be associated with either no or a slight increase in the risk of LPD development[7-10]. However, an increase in rates of LPD development in those with IBD who are on immunosuppressive therapy has been noted by different groups worldwide, documented in a variety of population-based, retrospective and case control studies[8,11,12]. Collectively, these studies point to the possibility that increased malignancy rates may be due the use of particular immunosuppressive therapies that inhibit normal host immunity and exposure to EBV, which in an immunosuppressed host, can infect host cells and result in malignant transformation. While limited data is available in the IBD population, there is a wealth of studies conducted on PTLD and rheumatoid arthritis patients. The development of PTLD primarily involves either reactivation of latent EBV infection or new EBV infection and as such, the development of PTLD is screened for in the most high-risk population (EBV negative recipient matched with EBV positive donor) by monitoring EBV viral load. In the rheumatoid arthritis population, the use of methotrexate is well described to confer a significant risk of lymphoma development. In this review, we first describe the well-established causal relationship between EBV infection and LPD development. Second, we explore the effect of immunosuppression, including biologics, in the post-transplantation and rheumatoid arthritis populations on EBV-associated LPD development. Third, we examine what is known currently about the risk of EBV-associated LPD development in patients treated with immune suppressants in IBD. Lastly, we discuss what can be translated from the post-transplantation literature to IBD to manage risks of EBV-associated LPD while on immune suppressants.

EBV CAUSES LPD

EBV is a double-stranded DNA virus belonging to the herpesvirus family that is ubiquitously found worldwide in roughly 90%-95% of adults[13]. The peak incidence by age is bimodal as roughly half of children under five years of age in developed countries acquire this relatively benign infection, often passing as a constellation of unremarkable upper respiratory tract infection symptom

while the second peak of infections occurs in the 15 to 24 years old group[13]. EBV spreads *via* oral secretions and blood, capable of triggering B-lymphocyte and epithelial cell uptake[13]. Once intracellular, EBV initiates the lytic phase of infection, resulting in the lysis of the cellular host and subsequent release of viral progenies. In an immunocompetent host, cell-mediated immunity is activated as cytotoxic T lymphocytes (CTLs) target viral infected cells for apoptosis[13]. A proportion of EBV infected B-lymphocytes escape CTLs detection and continues on to become long-lived infected memory B-lymphocytes where the virus persists in the latent phase of its life cycle[13].

In latent phase, viral proteins are capable of initiating host malignant transformation in a subset of individuals, resulting in uncontrolled memory B-lymphocytes proliferation, or a LPD. A number of prospective and case-control studies worldwide have identified EBV infection as a risk factor to the development of LPDs such as Hodgkin lymphoma, Burkitt's lymphoma, and a subset of aggressive non-Hodgkin lymphomas[14,15]. Hodgkin lymphoma has been the best studied and remains the lymphoma with the strongest association between EBV[16]. A causal relationship has been established in *in vitro* studies where EBV infection of human B-lymphocytes results in the uncontrolled proliferation of infected cells[17]. One study suggests that the rate of malignant transformation in EBV infected individuals occurs at a rate of 1:1000 over the span of four years from infection to Hodgkin lymphoma detection[14].

Certain risk factors have been associated with higher rates of LPDs. A case-control study from England revealed that the age of first infection is associated with a higher odds-ratio of developing Hodgkin's lymphoma with the highest odds-ratio in the 16-24 years of age group[18]. Immune deficient patients have increased susceptibility to LPD development in part due to an inability to mount an EBV-specific immune response. Those with compromised immunity[19] or those receiving immunosuppressive therapies[20,21] have been found to have an altered humoral immunity against EBV. As such, increased rates of EBV-associated LPD have been documented in patients with human immune-deficiency virus[22] those with inherited immune-deficiencies[23] and in post-transplanted patients receiving immunosuppressive therapy[24].

EFFECT OF POST-TRANSPLANTATION IMMUNOSUPPRESSION ON EBV-ASSOCIATED LPD DEVELOPMENT

EBV is thought to be responsible for the majority of cases of PTLD, defined as uncontrolled lymphoid or plasma cell proliferation post solid organ or hematologic transplantation in the setting of immunosuppressive agents[25]. PTLDs include a range of subtypes. Early lesions, which include plasmacytic hyperplasia and infectious mononucleosis, and polymorphic PTLD typically involve EBV and occur within the first year post transplant. On the other hand, monomorphic PTLDs, which are histologically identical to B- or T-cell derived non-transplant malignant lymphomas, tend to occur late post transplant, involve EBV less often, and are clinically more aggressive. Hodgkin lymphoma type PTLD is the least common subtype[26-30]. Similar to the risk factors for development of LPDs in immunocompetent patients infected with EBV, studies of post-transplant patients revealed the key risk factors for developing PTLD include the degree of T-lymphocyte immunosuppression and the EBV serostatus[31,32]. The risk of PTLD in renal transplant patients is thought to be 6-20 times higher than the general population while those receiving heart transplants have an estimated 200 times higher risk due to the relatively intensive immunosuppression that thoracic transplant recipients receive[29]. A number of multi-national retrospective database review studies revealed the greatest yearly incidence rate was seen in the first year post transplantation with the number of new cases steadily declining over the five years of study[32,33], suggesting that the degree of immunosuppression, which typically is highest during the first year post-transplantation, may increase the risk for PTLD development[29]. Studies with longer follow-up, however, show a second peak in incidence at around 8 years post-transplant, suggesting that prolonged high doses of immunosuppression are also associated with increased rates of PTLD development[30,34].

Different immune-suppressive induction agents have been hypothesized to confer different risk for developing PTLD[29,35,36]. In addition, combination therapy, while most successful at preventing rejection, is associated with greater risk of PTLD development in one pediatric population[37]. Agents that suppress CTLs, such as belatacept and efalizumab[38-40], and T-lymphocytes in general, such as OKT3 and thymoglobulin[36,38], were suggested to have a greater role in inducing PTLD than those that mediate general immunosuppression. Given that viral infected cells are cleared by activated CTLs, agents that hamper CTLs is thought to be permissive for viral infection and later malignant transformation of the infected host. The rates of PTLD were found to increase dramatically as well with the initial use of cyclosporine[41,42]. Fortunately, by implementing drug-level monitoring and dose reduction, rates of PTLD have dropped since the early days of cyclosporine use. Certain agents, such as mycophenolate mofetil, have not been associated with any increased risk of PTLD[38,43].

In addition to the degree and type of immunosuppression, EBV seronegativity is an independent risk factor for the development of PTLD. The risk of PTLD is greater in EBV seronegative patients who become infected while immune suppressed than in seropositive recipients reactivating latent EBV infection post-transplantation[24]. Numerous studies have identified EBV seroconversion after either solid organ or hematological transplantation as a risk factor for PTLD development[37,44-48]. EBV naïve patients receiving immune

suppressants were found to be at a higher risk of developing PTLD compared to EBV positive patients in one landmark University of Alberta retrospective study[49]. Since then, others in different centers have likewise identified EBV seronegativity in the pre-transplantation individual as a significant risk factor for developing PTLD post transplantation[44,45,50]. EBV seronegativity has a stronger impact on the risk of PTLD that occurs early (*i.e.*, within 1 year) as opposed to late post transplant[30,34]. As such, EBV seronegative patients are subjected to monitoring during the first year post-transplantation to detect PTLD development (discussed in the "PTLD Prevention" section).

PTLD prevention

To address the increased risk of malignant transformation of PTLD in context of immunosuppression and EBV infection, some have recommended that routine monitoring of EBV viral load be undertaken in the post-transplant settings. Rising viral load raises the suspicion of PTLD development since a high EBV viral load has been documented in some studies to precede the development of EBV-mediated PTLD[51-55]. As such, the absolute viral load has been proposed as prognostic of PTLD development[47,53]. However, in part due to a number of technical challenges of the EBV viral load assay, including a lack of standardized reference ranges for instrument calibration across multiple assay platforms, the positive predictive value of this assay remains low as an elevated viral load has high sensitivity but lacks specificity for PTLD development[56-61]. Thus, the utility of EBV viral load monitoring in a seropositive patient remains highly controversial[62-64]. On the other hand, serial EBV viral load monitoring in the seronegative recipient is an effective tool to identify those at clear risk of developing PTLD[65,66]. By routine monitoring of EBV viral load in the seronegative recipient, pre-emptive interventions, such as anti-viral treatment and rituximab therapy, may be undertaken to prevent PTLD development when rising EBV viral load is detected[55,65,67-71].

EBV-ASSOCIATED LPD DEVELOPMENT IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH IMMUNE SUPPRESSANTS

Several large-scale population studies have demonstrated a mildly elevated risk of LPD and EBV-associated LPD in those with rheumatoid arthritis (RA) and an even higher risk in patients being actively immunosuppressed compared to the healthy population[72-74]. Mechanistically, patients with RA have been shown to have defective EBV-specific T cell function, resulting in a greater number of infected lymphocytes and as such are at a higher risk than the general population for development of LPD[75]. The addition of an immunosuppressive agent further elevates the risk of EBV-associated LPD, increasing the relative risk for LPD development from 2.5 (RA without immunosuppression) to 10 (RA with immunosuppression)[76]. The highest incidence of LPD development typically occurs within the first year post treatment[77,78]. Various immune suppressive agents have been linked to an increased risk of malignancies. The best-studied immune suppressant in context of RA and LPD development is methotrexate (MTX). This immunosuppressive agent has such a strong association with LPD development that the 2008 World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues recognized MTX-associated LPD as in independent entity[79]. MTX-associated LPD is commonly characterized by the presence of EBV virus in the lymphoma tissue and that discontinuation of MTX in results in regression of LPD in many but not all patients[80,81]. Furthermore, this risk of MTX-associated LPD increases with higher treatment doses[82]. Thus, it has been proposed that should MTX treated RA patients develop LPD, they should then receive EBV serologic screening to determine if MTX should be discontinued[83] as the likelihood of regression post MTX discontinuation appear to be linked to EBV status[80].

Anti-tumor necrosis factor (TNF)α antibody therapy has also been shown to increase rates of LPD in RA patients compared to healthy controls in a systematic meta-analysis[84]. In one head to head comparison of anti-TNFα (infliximab) against MTX treatment, anti-TNFα agents were associated with higher rates of LPD than MTX[85]. The risk of LPD development is likewise correlated with higher doses of anti-TNFα (either infliximab or adalimumab)[84]. One recent report has even linked adalimumab to EBV-associated lymphoproliferative disorder development after two years of treatment[86].

EBV-ASSOCIATED LPD DEVELOPMENT IN IBD PATIENTS TREATED WITH IMMUNE SUPPRESSANTS

EBV and IBD

Given the extensive data from the post-transplantation and RA literature that shows the risk of EBV-associated LPD increases with immunosuppression, one logical question to ask is whether similar immunosuppression in other disease states, such as IBD, is also associated with increased rates of LPD. Furthermore, given that IBD patients are often diagnosed and initiate treatment younger than 30 years of age (an age demographic with many unexposed to EBV), concern for LPD risk in EBV seronegative patients may be raised.

Even without treatment, IBD patients have higher rates of infections such as non-antibiotic associated *Clostridium difficile* colitis[87], cytomegalovirus[87,88], and infectious colitis[88]. It is unclear why IBD is associated with higher rates of these specific infections. Regardless, it is clear is that with the addition of immunosuppressive agents, this infectious risk increases substantially[88-91]. However, with respect to EBV, Reijasse *et al*[92] did not

find a relationship between EBV viral load and severity of CD activity or the type of immunosuppression (infliximab infusion, corticosteroids, azathioprine and cyclosporine) used. Similarly, Fernandez Salazar *et al*[93] found that EBV seropositive CD patients in remission maintained on either no immunosuppression, azathioprine and/or infliximab did not have any significant changes to the viral load. However, interestingly patients with the most severe uncontrolled CD activity were found to have transient dramatic spikes in EBV viral load[92,94] with EBV DNA detected in colonic mucosal B-lymphocytes[94].

EBV-associated LPD and IBD

The link between EBV-associated LPD and IBD remains somewhat controversial as there have been a number of conflicting major studies done to date. The majority of large population-based studies have failed to find a significant association between IBD and LPD[95-100]. On the other hand, studies based in tertiary referral centers, which may have an inherent a referral bias towards those with more severe disease, showed that after factoring in the type and dose of immune suppressants used, IBD by itself does confer a slightly elevated risk for LPD[7,101]. In addition, when subgroup analysis was undertaken in one population-based study from the University of Manitoba, an increased risk of LPD in male patients with CD was found[102]. The difficulty in large population studies is that a number of factors, such as IBD disease severity and immune suppressant usage, are often not accounted for. Thus, it remains unclear how much IBD by itself, without the influence of immune suppressants, contributes to LPD development.

While the influence of IBD on EBV-associated LPD development has not been independently determined, analysis of IBD patients on immunosuppressive therapy demonstrates a clear risk for the development of EBV-associated LPD. An estimated 50% of IBD patients on immunosuppressive therapy with LPD were EBV seropositive[8,103] with a number of case reports identifying EBV DNA present in LPDs that developed post immunosuppression in IBD patients[104-107]. In reviewing the post-transplantation literature, a major risk for the development of PTLD is EBV seroconversion or EBV naivety while on immune suppressants and those who were EBV seropositive prior to transplantation habouring a latent infection represents a minor risk factor[44,45,49,50]. Currently, only a handful of case reports have documented the link between EBV seroconversion and LPD development in context of IBD and immunosuppression. Van Biervliet *et al*[108] reported the case of a young EBV seronegative CD patient who developed LPD shortly after treatment with azathioprine. Similarly, a 16-year-old CD patient who became seropositive while on therapy of mesalamine, azathioprine and infliximab infusion consequently developed EBV-associated LPD[109]. Lastly, a 25-year-old CD patient develops LPD after undergoing EBV seroconversion while on azathioprine[110]. Taken together, these reports may indicate a risk of LPD development from EBV seroconversion while on immune suppressants in IBD patients on immune suppressants. Perhaps an argument can thus be made for EBV serological monitoring in the EBV naïve IBD population. However, more research is needed to determine the effectiveness and utility of such an approach.

Role of immune suppressants on EBV-associated LPD development in IBD

Medical therapy for IBD is often individualized and there are nuanced differences between the management of CD and UC[111]. Regardless, typical immunosuppressive regimens may include prednisone, mesalazine, cyclosporine, thiopurines, such as azathioprine (AZA) and 6-mercaptopurine (6-MP), and infliximab[111]. (MTX is also used in IBD treatment, though much less frequently than in RA management and thus scant safety data is available in the IBD population). These therapies have been examined for a correlation with EBV-associated LPD development. Mechanistically, it is theorized that increased cancer risk may be conferred with a disturbed mucosal barrier and increased inflammation resulting in an accumulation of genetic mutations provides the opportunity for EBV-mediated malignant transformation. Immunosuppressive agents hamper the innate and adaptive responses for tumor surveillance and clearance[95].

The best studied of all IBD treatment agents, AZA and 6-MP, were associated with an increased risk of LPD when standard dosing (AZA 2.5 mg/kg per day; 6-MP 1.5 mg/kg per day) were used[8,10-12]. Dayharsh *et al*[112] found in a retrospective study that thiopurine use dramatically increased the rates of EBV-associated LPD in their IBD population (17% increase to 50%). Similarly, thiopurine treatment in a French nationwide prospective observational cohort study (CESAME) was associated with increased EBV-associated lymphomas[113]. A recent review of the Kaiser Permanente Cancer Registry of 16023 IBD patients revealed an increased incidence of lymphomas in thiopurine treated patients[9]. Finally, in a recent meta-analysis[114] and a retrospective cohort study of the United States Veteran Affairs database[11], both publications demonstrated a 4-fold increased risk of lymphoma in AZA or 6-MP treated IBD patients compared with the general population[11,114]. The meta-analysis found the lymphoma development risk increased with duration of immunosuppression and decrease with discontinuation of therapy[114]. In fact, one case report described lymphoma regression upon withdrawal of thiopurine[115]. Thus, given the higher risk of EBV-associated LPD development in young male IBD patients, some groups have proposed the avoidance of thiopurine use in this particular population altogether[116,117].

In addition to thiopurine, other IBD treatment agents have been studied, albeit to a lesser extent. MTX is one such agent. There has been scant data on MTX and EBV-associated LPD development in the IBD population. Kandiel *et al*[11] found that 2 of the 4 cases of lymphoma development in IBD patients involved treatment with MTX (31 patients of the 782 person study

received MTX in total). While studies in IBD are lacking, studies involving patients with rheumatoid arthritis found MTX treatment to be associated with increased risk of lymphoma development[118,119]. One case report documented the development of EBV-associated LPD in a patient with rheumatoid arthritis receiving MTX with lymphoma regression upon discontinuation of MTX use[120].

Another commonly used class of IBD agents is the anti-TNFα antibody, including both adalimumab and infliximab[12]. Adalimumab has been linked to Hodgkin Lymphoma development[121] or recurrence[122]. However, the largest trial to date involving adalimumab use found no increased incidences of T-cell non Hodgkin Lymphoma development over control[123]. This study, however, did find increased risk of T-cell non Hodgkin Lymphoma development in those treated with anti-TNFα agents (either adalimumab or infliximab) in combination with a thiopurine[123]. The link between infliximab and LPD is likewise controversial. There are a number of trials that have identified a small but significant risk of lymphoma development in IBD patients on infliximab. In the ACCENT Ⅰ maintenance infliximab infusion randomized placebo-controlled trial, two cases of EBV-associated non-Hodgkin lymphoma were found out of 573 patients (all participants had a score of at least 220 on the CD activity index)[124]. A second study based at the Mayo Clinic found one case of EBV-associated lymphoma out of 500 patients[11]. A third smaller randomized, double-blinded placebo controlled trial of 73 IBD patients who were either refractory to conventional treatments or responded sub-optimally to treatment were initiated on a course of four infliximab infusions every 8 wk[125]. One patient developed B-cell lymphoma 9.5 mo post initial infusion[125]. A large retrospective chart review of the Kaiser Permanente Cancer Registry revealed an increased standardized incidence rate ratio (5.5 for past use; 4.4 for current use) of lymphoma development over nearly 6-year span in the IBD population treated with infliximab over those without[9]. Finally, a recent meta-analysis of 26 publications found, in subgroup analysis, an increased risk of non-Hodgkin's lymphoma development in anti-TNFα agent treated IBD male patients aged 20-54 years of age[12]. On the other hand, a number of studies have failed to find evidence of increased LPD risk from infliximab use. The large Crohn's Therapy Resource, Evaluation, and Assessment Tool registry found no increased risk of lymphoma in IBD patients treated with infliximab over control population[126]. A selective small meta-analysis of randomized controlled trials failed to find an increased risk of lymphoma associated with infliximab over those that did not receive any anti-TNFα agents. Finally, a recent study of long-term safety of infliximab use found no increase LPD risk conferred by infliximab over control over the span of 14 years[127].

There are several inherent difficulties in establishing a role for infliximab in EBV-associated LPD development in IBD. First, most studies do not stratify the data based on disease severity. It may be reasonable to suspect that those requiring treatment with an anti-TNFα agent is associated with more refractory or severe disease as biologics are typically prescribed after other immune suppressants have failed. As such, more severe inflammatory disease may independently confer a higher LPD risk. Second, it maybe challenging to show the effect of anti-TNFα therapy alone in the development of LPD as the control group typically has received some form of immunosuppressive therapy. Third, very few patients will have received only anti-TNFα therapy without prior exposure to any other immunosuppressive agents. As such, there may be an accumulated risk from multiple agent use. This raises the hypothesis that it may not be any specific immunosuppressive agent that may be the culprit for LPD development, but rather the combination or addition of the third or the fourth agent that statistically increases LPD risk[128]. One observation that supports this theory is the increasing rates of hepatosplenic T-cell lymphoma (HSTCL) where the majority of reported cases involve young male patients (average age mid-twenties) receiving either prolonged thiopurine therapy (more than two years) or combination immunosuppression therapy of thiopurine and anti-TNF therapy[129,130]. As such, some have proposed that male patients under 35 years of age on prolonged thiopurine treatment or combination therapy should be monitored carefully for signs of HSTCL[129,130].

In summary, EBV-associated LPD may not be elevated in IBD from a population perspective but appears to occur more frequently in the younger male population, possibly due to the fact that significant EBV exposure occurs during this time. What might be behind the gender differences is currently unclear. In addition, regardless of patient demographics, thiopurines appear to confer the greatest risk of EBV-associated LPD development when compared to the methotrexate or biologics.

LESSONS FROM IMMUNE SUPPRESSION USE: FUTURE DIRECTIONS FOR IBD RESEARCH

Attempting to interpret findings from one field and apply them to another must be done with caution, as the dosing and treatment regimens of immune suppressants used in IBD are different than those used post-transplantation or in RA. Furthermore, the pathophysiology of these diseases, although incompletely elucidated, are likely quite different. However, given the sparse data available in the IBD field surrounding the risks of immune suppressants, complications from their use in the context of other inflammatory diseases should also not be overlooked. Currently, there is a trend amongst IBD physicians to move towards increased use of MTX for the purposes of both primary immunosuppression and also for suppression of anti-biologics antibody production. As the data linking lymphoma risk in MTX use in RA is mounting, the role of MTX in lymphoma development

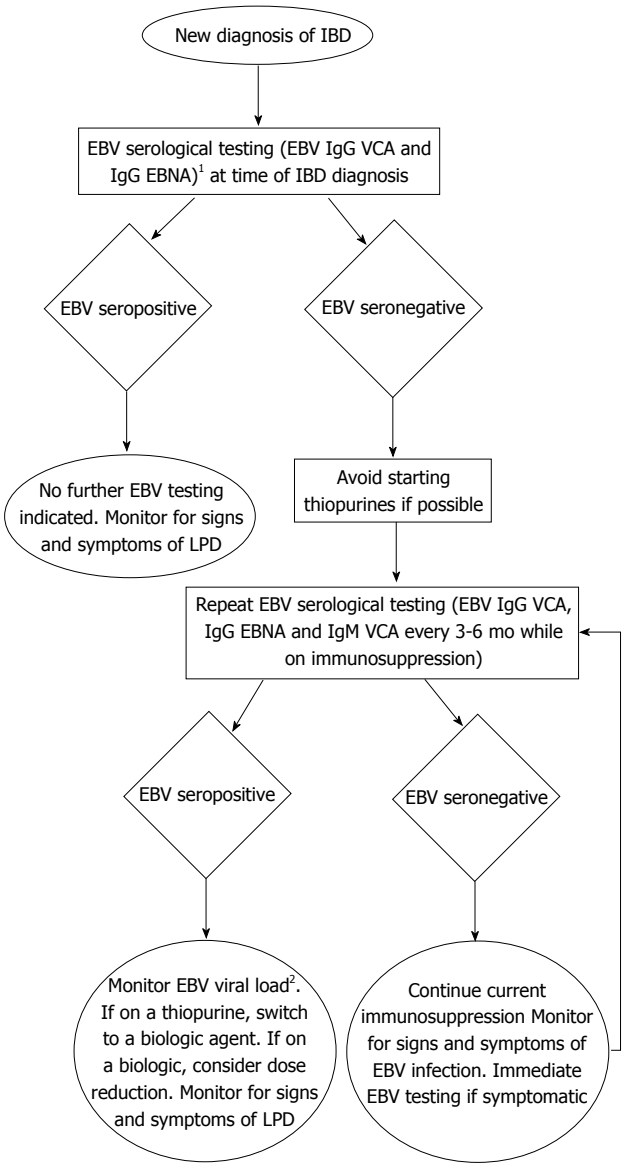

Figure 1 Proposed algorithm for treatment management of inflammatory bowel disease patients who are either Epstein-Barr virus seropositive or negative. [1]EBV monospot or EBV IgM have not been shown to be helpful in in determining serostatus; [2]EBV viral load should be done by polymerase chain reaction in whole blood in EDTA collection tube. EBV: Epstein-Barr virus; IBD: Inflammatory bowel disease; EBNA: Epstein-Barr virus-determined nuclear antigen; LPD: Lymphoproliferative disorders; VCA: Viral-capsid antigen; EDTA: Ethylenediaminetetraacetic acid.

in IBD should be examined more closely. Furthermore, it remains largely unclear what effect the dose, the combination and the duration of IBD immunosuppressive therapy has on EBV-associated LPD development. In review of the available data, more questions remain than answers. Is there a role for EBV serological screening as in the post-transplantation field and if so, who should be screened and for how long? The younger male population appears to have a higher risk of LPD development while on immune suppressants and given the second peak of EBV seroconversion is within the same age range, should males between the ages of 18 to 30 be selected for routine EBV viral load screening while on therapy? Are there certain combinations of drugs or specific therapies that should be avoided or dose adjusted to minimize the risk of EBV-associated LPD? Should withdrawal of immunosuppressive therapy be initiated as soon as metrics of early remission is achieved to minimize LPD risk? How should this be balanced with the risk of disease flare or risk of subsequent surgery? The benefit of immunosuppressive therapies in IBD, much like in RA, is unequivocal but the risk of LPD development is a cost that while relatively small is one which not all patients are comfortable with. Many questions surrounding how best to utilize and discontinue these powerful immunosuppressive agents remain. As such, the development of an early screening tool to further minimize the risk of LPD may invaluable to all IBD patients on immunosuppressive treatment (Figure 1).

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REVIEW

Current understanding of the neuropathophysiology of pain in chronic pancreatitis

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Abstract

Chronic pancreatitis (CP) is a chronic inflammatory disease of the pancreas. The main symptom of patients with CP is chronic and severe abdominal pain. However, the pathophysiology of pain in CP remains obscure. Traditionally, researchers believed that the pain was caused by anatomical changes in pancreatic structure. However, treatment outcomes based on such beliefs are considered unsatisfactory. The emerging explanations of pain in CP are trending toward neurobiological theories. This article aims to review current evidence regarding the neuropathophysiology of pain in CP and its potential implications for the development of new treatments for pain in CP.

Key words: Neurobiology; Neuropathophysiology; Pain; Pancreatic pain; Chronic pancreatitis

Core tip: Abdominal pain is the main symptom of patients with chronic pancreatitis (CP), yet the underlying mechanisms are not well understood. The emerging explanations of pain in CP are trending toward neurobiological theories. This article reviews these emerging concepts and their potential implications for the development of new treatments for pain in CP. Three major concepts attempting to explain the pathogenesis of CP pain: Pancreatic nociception and sensitization-induced pain, neuropathic remodeling, and central mechanism of pancreatitis pain are summarized, along with the specific molecules involved in each and potential therapeutic targets.

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INTRODUCTION

Chronic pancreatitis (CP) is a persistent and chronic inflammatory disease of the pancreas. Approximately,

80%-90% of patients with CP typically suffer from pancreatic pain[1], which is commonly described as a constant, severe, and dull pain in the mid-epigastrium that radiates to the back and worsens by with high-fat meals. Unsurprisingly, the pancreatic pain can have substantial psychological and economic impact on patients. In addition, a recent study confirmed that the life quality of patients with CP is significantly worsened by pain severity and disease-related complications[2].

The pathogenesis of pancreatic pain is still not fully understood. Thus, management of this pain cannot be specific, leading to unnecessarily high treatment costs and ineffective outcomes. Many theories have been proposed to explain the pain mechanism based on anatomical changes including high pressure within the pancreatic duct, high pressure in the pancreatic parenchyma, and complications of pancreatic and extra-pancreatic structures (*i.e.*, pseudocysts, duodenal and bile duct obstruction, and peptic ulcer). These anatomical changes are believed to be noxious stimuli that activate pancreatic pain *via* nociceptive pathways. However, a number of human studies of CP have demonstrated evidence against the above theories, finding, for example, no relationship between pain and pancreatic duct pressure reduction[3,4], no relationship between pain and increase of parenchymal pressure[5], no pancreatic duct dilation in some patients with severe pancreatic pain[6], and no relationship between pain and severity of CP-related structural changes on imaging[7]. Therefore, the pain of CP patients cannot be explained by mechanical stimulation of nociceptive pathways alone.

Since the late 1990s, investigators have been trending toward neurobiological theories to explain pain in CP[8]. Therefore, the main objective of this paper was to review the current neurobiological theories and emerging concepts that might lead to the development of new treatment regimens for alleviating pain in CP patients.

NEUROPHYSIOLOGY OF THE PANCREATIC PAIN

The pancreas is innervated by a complex structure of two groups of afferent fibers. The first group consists of branches of the abdominal vagus nerve, and the second fibers that run through the celiac plexus and reach the lower thoracic segments of the spinal cord *via* the splanchnic nerves[9]. The latter group is best known for stimulating visceral pain.

The nociceptive pathway in the pancreas begins with nociceptors located at the ends of the primary afferent neurons and function as afferent nerve endings[10]. Unlike those in other visceral organs, these primary afferent neurons convey only pain stimuli. One special subset of theses nociceptors contains a group called "silent nociceptors", which are only activated during inflammatory processes[11]. Furthermore, the pancreatic nociceptors can be activated by various noxious stimuli through mechanosensitive and chemosensitive mechanisms[12]. The former mechanism is located on blood vessels that supply the pancreas and pancreatic parenchyma and can be stimulated by stretching, ischemia, and necrosis. The latter mechanism can be stimulated by inflammatory mediators, but the exact location of this mechanism is not completely known.

The pathogenesis of CP is strongly related to prolonged exposure to noxious stimuli, which causes chronic inflammation. Noxious stimuli not only stimulate nociceptors, but can also damage pancreatic tissues and nerves surrounding the pancreas[13]. The injured tissues can release pro-inflammatory mediators such as prostanoid, bradykinin, tachykinin, serotonin, and growth factors[14]. Induced by the above mediators, primary sensory neurons then become more sensitive to further stimulation by either noxious (hyperalgesia) or non-noxious (allodynia) stimuli. This process is called peripheral sensitization[15], which indicates that the noxious stimuli can evoke nociceptor plasticity. Moreover, there is another mechanism by which pain can be exacerbated *via* peripheral sensitization, which begins with the activation of silent nociceptors by peripheral inflammation, and the silent nociceptors consequently facilitate and increase afferent activities in the spinal cord.

Once stimulated by pro-inflammatory mediators, the nociceptors will transform the stimuli into action potentials by unbalancing the Na and K currents on the neuronal membrane. The action potentials travel along both unmyelinated C-fibers and small myelinated Aδ fibers of primary sensory neurons[11,12]. These neurons traverse paravertebral and prevertebral ganglia to synapse with secondary sensory neurons at laminae Ⅰ, Ⅱ, Ⅴ, and Ⅹ of the dorsal horn of the spinal cord at the T5-L2 level. Based on an animal study, the secondary sensory neurons related to the pancreas are primarily located at the T10-T11 level[12]. Consequently, the primary sensory axons release glutamate, substance P, and calcitonin gene-related peptide (CGRP). Glutamate activates both α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and N-methyl-D-aspartate (NMDA) receptors, while substance P activates NK1 receptors[16,17]. These three receptors are located on secondary sensory neurons within the dorsal horn. At this level of stimulation, prolonged stimulation from peripheral sensitization can facilitate excitation of dorsal horn neurons, which can increase spontaneous activities, decrease the firing threshold, and expand the receptive field of the dorsal horn neurons. This process is called central sensitization and can result in hyperalgesia and allodynia[11].

After the activation of secondary sensory neurons, action potentials are generated and transmitted to the thalamus *via* the spinothalamic tract to activate tertiary sensory neurons. These tertiary sensory neurons then transmit the signal to the somatosensory cortex for cognitive integration of pain and the limbic system and hypothalamus for autonomic/affective integration of the pain[18].

Furthermore, the central nervous system (CNS) can modulate pain signaling at the spinal cord level *via* either facilitation, increasing the spinal transmission of pain impulses, or inhibition, decreasing the spinal transmission of pain impulses. The combination of facilitation and inhibition generates the signal that will determine the pain perception in the brain.

After the primary sensory neurons are activated, neurotransmitters (glutamate, substance P, and CGRP) are not only released to the dorsal horn of the spinal cord, but also to primary nerve endings located on the pancreas, where they act as inflammatory mediators that create pancreatic inflammation characterized by vasodilation, edema, and neutrophil infiltration. This process is also known as neurogenic inflammation[19-21]. Additionally, this neurogenic inflammation can facilitate the activation of peripheral sensitization[10].

NEUROPATHOPHYSIOLOGY OF PANCREATIC PAIN

Chronic inflammation in the pancreas has been shown to spread to the pancreatic nerve[22,23]. Additionally, perineural inflammatory cells including eosinophils, $CD4^+$ and $CD8^+$ lymphocytes, macrophages, and mast cells are evidenced in patients with painful CP[24-27]. This finding is consistent with the increased percentage of eosinophils observed in perineural inflammatory cell infiltrates, which may be related to the release of a nociceptive substance[13]. In addition, numerous studies[28-34] have reported the increase of various perineural inflammatory mediators including histamine, serotonin, interleukin, bradykinin, substance P, CGRP, tumor necrosis factor-alpha, and several neurotrophins [*i.e.*, growth-associated protein 43, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF)]. Specifically, BDNF and NGF up-regulation has been shown in CP patients[24,26].

Such evidence has recently become the main focus of many studies attempting to explain the pathogenesis of pain based on three concepts: pancreatic nociception and sensitization-induced pain, neuropathic remodeling (neuropathic pain), and central mechanism of pancreatitis pain. Each of these aspects is complex and involves specific molecules that are described in the following sections.

PANCREATIC NOCICEPTION AND SENSITIZATION-INDUCED PAIN

There is much evidence to support that peripheral and central sensitization is largely associated with the pancreatic pain in CP. The evidence related to the molecules and receptors that have been found to be involved in the sensitization mechanisms will be discussed one-by-one in the following paragraphs.

The transient receptor potential (TRP) family is a group of ion channels localized mainly to the plasma membrane of neurons. Three molecules strongly related to pain and inflammation in the TRP family are TRP vanilloid 1 (TRPV1), TRPV4, and TRP ankyrin 1 (TRPA1)[35]. These three TRP channels are also associated with pain in CP patients through the sensitization of pancreatic afferent neurons and development of neurogenic inflammation. The primary sensory nerve endings that supply the pancreas contain these three types of TRP, which can be stimulated by specific stimuli including inflammatory mediators. After the receptors are stimulated, primary sensory neurons then release substance P and CGRP at both the spinal cord and peripheral sites, thus causing pancreatic inflammation *via* neurogenic inflammation[36-40]. The mechanism of peripheral sensitization (Figure 1) is discussed below.

TRPV1

TRPV1 can be directly activated by many factors, including heat, extra-cellular proton and tissue acidosis, capsaicin, biologically active compounds (anandamide and hydrogen sulfide), and endogenous lipid metabolites from the arachidonic acid pathway[41,42]. Furthermore, TRPV1 can be indirectly activated by pro-inflammatory bradykinin and pro-inflammatory leukotriene[43]. By modulating TRPV1 activity, pro-inflammatory bradykinin can indirectly activate TRPV1 *via* B2 receptors residing on primary sensory neurons. By binding to their leukotriene B4 receptors, pro-inflammatory leukotriene B4 can activate TRPV1 *via* an intra-neural signaling pathway. Furthermore, pro-inflammatory agents can sensitize TRPV1 by reducing the threshold of thermal stimuli (hyperalgesia)[44].

In animal and human studies, TRPV1 plays an important role in explaining pain in CP. After TRPV1 receptor activation by capsaicin in rats with induced CP, peripheral sensitization is evidenced by the significant upregulation of TRPV1 at both mRNA and protein levels in the dorsal root ganglion (DRG) and pancreas-specific sensory neurons[45]. Moreover, the same study found significant reduction of pain behavior and hyperalgesia after administration of a systemic TRPV1 antagonist. Significant upregulation of TRPV1 is also seen in the pancreatic tissue of patients with painful CP; however, no relationship was found between the pain score level and the level of TRPV1 expression[46].

TRPA1

TRPA1 is responsive to various stimuli that can be categorized into five groups: The pungent ingredients of spices, environmental irritants, endogenous agonists of TRPA1[39], cyclopentenone prostaglandins, and general anesthetics[47]. The pungent ingredients of spices include mustard oil[48], garlic[48], and cinnamon[48,49], and environmental irritants include acrolein[48,50], formaldehyde[48,51], and cigarette smoke[36,48]. Cyclopentenone prostaglandins include PGA2, PGA1, and PGJ2[52,53]. Pro-inflammatory agents also sensitize TRPA1 leading to hyperalgesia[54-56].

TRPV4

TRPV4 responds to changes in tonicity[57,58], moderate

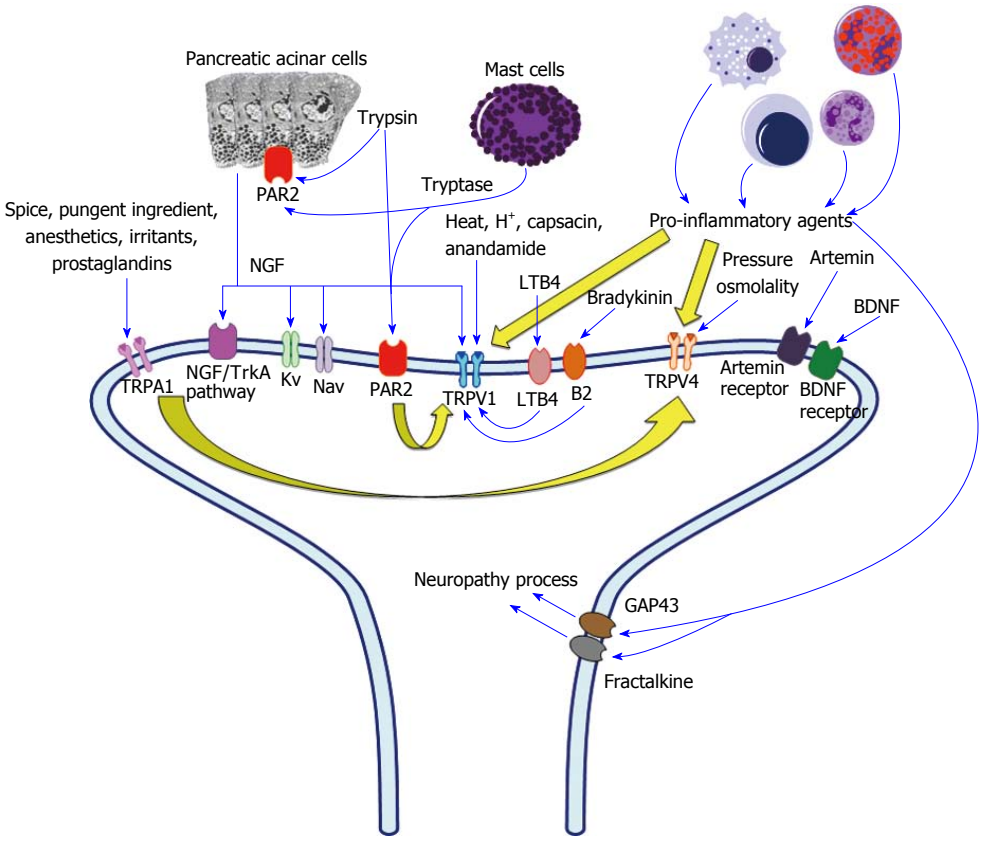

Figure 1 The mechanism of peripheral sensitization. PAR2: Proteinase-activated receptor 2; NGF: Nerve growth factor; TRPA1: Transient receptor potential ankyrin 1; TRPV1: Transient receptor potential vanilloid 1; BDNF: Brain-derived neurotrophic factor; GAP43: Growth-associated protein 43; LTB4: Leukotriene B4.

heat (> 27 ℃)[37], and mechanical pain[37]. Changes in tonicity can cause cell swelling and activate phospholipase A2; this process leads to the generation of arachidonic acid[59], which is an endogenous agonist of TRPV4. In addition, 4α-phorbol 12,13-didecanoate (4αPDD) is a synthetic TRPV4 agonist[60,61]. Similar to TRPV1 and TRPA1, pro-inflammatory agents can sensitize TRPV4 causing hyperalgesia to mechanical stimuli[62-64].

To the best of our knowledge, the first evidence that TRPA1 and TRPV4 contribute to pancreatitis pain was reported in rats with induced acute pancreatitis[48]. Another study also demonstrated that TRPA1 mediates CP pain in mice[54]. In a recent study using mice in which CP was induced through repetitive cerulein injections, TRPV1 and TRPA1 antagonists were important in alleviating neurogenic inflammation in pancreatitis, reducing pain-related behavior, and preventing the transition from acute to chronic inflammation[65]. Therefore, TRPV1, TRPA1, and TRPV4 are likely to be targets for therapeutic pain management in CP patients by reducing peripheral sensitization and neuropathic inflammation.

Proteinase-activated receptor 2

Proteinase-activated receptor 2 (PAR2) is one of the chief regulators of pancreatic exocrine secretion in pancreatic acinar cells and ductal epithelium. Notably, trypsin is recognized as the strongest activator of PAR2. There is also evidence supporting a relationship between PAR2 and pancreatic pain. PAR2 expression was detected in sensory neurons supplying the pancreas; in fact, primary sensory neurons could be activated and sensitized by administering PAR2-specific proteinase activating peptide and trypsin in an *in vivo* study[66,67]. Moreover, both PAR2-specific proteinase activating peptide and trypsin-induced behavioral pain response have been observed in awake rats[67]. Another study discovered that tryptase, a substance released from activated mast cells, can stimulate PAR2[27], which might explain the relationship between mast cells and pain in CP patients. In an experimental animal model of pancreatitis pain, the administration of two proteinase inhibitors (camostat mesylate and nafamostat mesylate) reduced sensitivity to abdominal pain[68]. Likewise, nafamostat was associated with a significant reduction of pain duration induced by acute pancreatitis[69].

Based on *in vitro* findings, PAR2 activation causes TRPV1 sensitization by enhancing capsaicin; consequently, this process leads to the significant release of CGRP[70]. Similarly, in *in vivo* studies, PAR2 activation resulted in pain-related behavior[55,70,71]. As additional supporting evidence that PAR2 is involved in the development of hyperalgesia, PAR2 was significantly upregulated in DRG neurons along with decreased thermal withdrawal latencies in a rat model of CP[72]. In short, PAR2 agonist peptides, trypsin and tryptase, are related to the pathogenesis of pain in CP *via* nociception and sensitization caused by the interaction between TRPV1 and PAR2.

NGF

NGF, a type of neurotrophin, is a protein important for the growth, maintenance, regulation of survival, and specialization of sensory neurons. Moreover, NGF is an essential mediator of peripheral sensitization[73]. Although the islets of pancreatocytes typically generate NGF, NGF was found to be upregulated and surprisingly expressed in pancreatic acinar cells and ductal epithelium in a rat model of pancreatitis[74]. However, the upregulation of NGF returned to normal after the pancreatic inflammation resolved[16]. Many studies have attempted to explain the mechanism of NGF-induced pancreatitis pain on sensitization *via* modulation of TRPV1 and excitability of K and Na currents[73,75-77]. Another hypothesized mechanism underlying pain in CP is activation of the NGF/trkA pathway[78,79]. In a study of rats with CP induced by trinitrobenzene sulfonic acid, both anti-NGF antibodies and trkA-immunoglobulin G substantially reduced hyperalgesia[80,81].

Artemin

Artemin is a neurotrophin classified as a glial cell line-derived neurotrophic factor. Overexpression of artemin and its co-receptor GFR alpha 3 has been reported to strongly relate to the increased frequency and intensity of pain in rats with CP[82].

BDNF

BDNF is also a member of the neurotrophin family found in the brain and periphery. An *in vivo* study reported that BDNF is upregulated in primary sensory neurons in rats with CP, and that BDNF antagonist treatment was associated with a reduction of pain-related behavior in these animals[83]. Another study of pancreatic tissue in patients with CP found that pain was positively related with BDNF levels and increased in CP patients compared to healthy control. These findings suggest that BDNF is essential to the nociceptive pathway of CP.

Other substances

Studies have also reported associations between pain in CP and other substances that could be related to peripheral sensitization, for example, the over-expression of interleukin 1[84], interleukin 6[85], interleukin 8[86], and fractalkine[87].

Neurotransmitter expression

Previous findings in patients with painful CP indicate overexpression of neurokinin 1[88], neurokinin 2[88], CGRP[16], and substance P[16,88]. Therefore, overexpression of these neurotransmitters may result from activation of nociceptive pathways and peripheral sensitization.

PANCREATIC NEUROPATHIC REMODELING-INDUCED PAIN

In clinico-pathological studies, the intra-pancreatic nerves in patients with painful CP demonstrate immune cell infiltration, indicating pancreatic neuritis[13,89], and characteristics of pancreatic neuropathy, which can be described as the increase of neural density, hypertrophy, and spouting[13,90-92]. Both pancreatic neuritis and pancreatic neuropathy are believed to relate with the inflammatory process, which is a key pathogenic factor in CP as indicated by the following evidence. The increase of fractalkine and its receptor is correlated with fibrosis, neuropathic changes, pain duration of CP and the degree of inflammatory cell infiltrate[87,91,92]. Moreover, the expression of growth-associated protein 43 (GAP43), which is a member of the neurotrophin family, is reported to have a relationship with pancreatic neuropathy, pancreatic neuritis, and pancreatic pain. Consequently, GAP43 may be considered a potential marker of neuronal plasticity during development and injury[87,89,91,92].

Patients with painful CP have been reported to demonstrate significant alterations in pancreatic innervation, with a marked decrease in sympathetic innervation but no statistically significant difference in cholinergic innervation[92]. In the same study, stronger expression of pain-related behavior was also noted in patients with painful CP, indicating neuronal regeneration after neuron injury.

In conclusion, the inflammatory process leaves pancreatic neurons damaged and characterized as showing either neuropathy or neuritis. Correspondingly, these neurons express GAP43, leading to the remodeling of pancreatic innervation. This process might explain pancreatic pain in CP patients. Such a process is similar to pancreatic nociception and sensitization-induced pain in the sense that both processes involve inflammatory mediators. However, the mechanism by which inflammatory mediators induce neuropathic pain is by destroying the neurons, leading to permanent neuronal lesions without involving noxious stimuli and the sensitization process.

CENTRAL MECHANISM OF PANCREATITIS-INDUCED PAIN

Central sensitization

As previously described, several factors can induce pain in CP by triggering the CNS, for instance, chronic stimulation of pain through nociceptive pathways, peripheral sensitization caused by inflammatory processes in the pancreas, and nerve damage. Consequently, prolonged peripheral sensitization can lead to central sensitization, which will be discussed next.

Using quantitative sensory testing in human experiments, researchers found that the brain activity of patients with CP demonstrated increased areas of referred pain and increased heterogeneity of referred pain location compared to the control group after electrical stimulation of the esophagus, stomach, and duodenum[93]. The sensitization caused by CP could decrease the pain threshold and increase the referred pain area[94,95].

Table 1 Potential treatment alternatives and their drug targets

Drug target	Potential treatment alternatives
NGF	Tanezumab
TRPV1	TRPV1 antagonist
PAR2	Trypsin inhibitors
Mast cell	Ketotifen
Interleukin 1	Recombinant interleukin-1 receptor antagonist
Interleukin 6	Interleukin-6 antagonist
Central sensitization	Ketamine, dextromethrophan, pregabalin, tricyclic antidepressants, and noradrenaline reuptake inhibitors

NGF: Nerve growth factor; TRPV1: Transient receptor potential vanilloid 1; PAR2: Proteinase-activated receptor 2.

By using electroencephalography (EEG) to measure brain activities, studies of pain in CP can be categorized as either resting-state EEG or evoked potential (EP) tests[84,96]. In resting-state EEG, alpha activities were found to demonstrate increased amplitude strength in CP patients compared to healthy volunteers[97], and pain duration was negatively correlated with the average peak alpha frequency[98]. Notably, the relationship between chronic pain and the change in alpha activity could be the result of thalamocortical dysrhythmia, which is activated by T-type calcium channels[99]. In EP tests, constant electrical stimulation of the upper gastrointestinal tract significantly decreased latencies of the early EP components in CP patients compared to healthy volunteers[93]. Moreover, hyperalgesia and prolonged latencies of early visceral EPs components in the frontal region of the cortex were seen following electrical stimulation in CP patients compared to healthy subjects[100].

As observed with functional magnetic resonance imaging, pain sensation is processed and localized in somatosensory cortex, insula, anterior cingulate cortex, prefrontal cortex, and thalamus. Recently reported evidence indicates that plasticity, *i.e.*, functional or structural changes, in the CNS may be associated with pain in chronic syndromes. The structural reduction of cortical thickness[101] and microstructural changes in the insula and frontal cortex[102] also have been observed in magnetic resonance imaging studies.

The above findings support the hypothesis that the pain experienced by CP patients can be triggered by central sensitization, which is derived from sustained and increased peripheral nociceptive drivers. Moreover, recent studies have demonstrated that descending inhibitory modulators are significantly impaired in patients with CP compared to healthy controls[95,103]. Descending facilitation from the brainstem was also reported to be a critical factor in pancreatic pain in rats with CP[20].

POTENTIAL APPLICATIONS

Generally, drug discovery involves finding a new drug with the ability to increase or decrease the activities of selected targets or unrelated targets. The greater our understanding of the neuropathophysiology of pain in CP, the better our opportunity to identify potential treatment alternatives. Currently, there are two groups of potential treatment alternatives and their drug targets, which are summarized in Table 1. The first group of potential treatment alternatives is directed at attenuating the peripheral sensitization process by targeting related molecules and receptors, such as NGF, TRPV1, PAR2, trypsin, tryptase, interleukin 1, and interleukin 6. The second group of potential treatment alternatives focuses on attenuating the central sensitization process.

Anti-NGF antibody demonstrated a significant effect on attenuating the changes in the excitation of pancreatic nociceptors in rats with CP[81]. Tanezumab, a humanized monoclonal antibody with specific binding to NGF, is able to relieve chronic pain in many conditions, for instance, chronic low back pain[8,104], interstitial cystitis[8,105,106], and osteoarthritis knee pain[8,107,108]. However, to the best of our knowledge, there has not been any human study to date using anti-NGF in CP.

A TRPV1 antagonist remarkably reduced both visceral pain behavior and referred somatic hyperalgesia in rats with CP[45]. Since not only TRPA1 but also TRPV4 are related to the peripheral sensitization of pain in CP, theoretically both TRPV1 and TRPV4 antagonists should be able to attenuate pain in CP. Nevertheless, we have not seen any study using a TRPV4 antagonist in CP.

Although PAR2 is the receptor that induces peripheral sensitization of pain in CP, direct PAR2 antagonists are very difficult to create[8]. As already mentioned, both trypsin and tryptase are agonists of the PAR2 receptor. Therefore, one researcher proposed that PAR2-sensitized pain can be inhibited indirectly by using trypsin inhibitors and a mast cell stabilizer (ketotifen)[8].

In the inflammatory process, interleukin 1 and interleukin 6 are associated with pain in CP. As a result, antagonists of both these interleukins may be able to attenuate pain. Researchers found that a recombinant interleukin-1 receptor antagonist[109] and interleukin-6 antagonist[85] can have an effect on attenuating pancreatitis-induced pain in rats with CP.

Central sensitization of pain in CP can be influenced by NMDA receptors, thalamocortical dysrhythmia, and impaired modulation pathways. Consequently, we can attenuate pain in CP by modifying the activities of these influencing factors. Several known drugs can reduce the effect of central sensitization, such as ketamine[8,110,111], dextromethrophan[8,112], pregabalin[113-115], tricyclic antidepressants[84], and noradrenaline reuptake inhibitors[84].

CONCLUSION

Chronic pain is an important issue that significantly lowers quality of life in patients with CP. The theories for underlying causes of pancreatic pain in CP have been shifting away from anatomical changes of pancreatic structure to changes in neurobiological structure, which include peripheral sensitization-induced pain, neuropathic remodeling, and central sensitization of

pancreatic pain. Furthermore, researchers have identified numerous molecules related to pancreatic pain in CP, for example, TRPV1, TRPA1, TRPV4, PAR2, NGF, artemin, BDBF, GAP43, and fractalkine. As a result, the neuropathophysiological mechanisms of pain in CP show strong potential as targets for drug discovery to relieve the pain and improve quality of life in this patient population.

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MINIREVIEWS

Faecal calprotectin: Management in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a chronic and relapsing disorder which leads to an inflammation of the gastrointestinal tract. A tailored therapy to achieve mucosal healing with the less adverse events has become a key issue in the management of IBD. In the past, the clinical remission was the most important factor to consider for adapting diagnostic procedures and therapeutic strategies. However, there is no a good correlation between symptoms and intestinal lesions, so currently the goals of treatment are to achieve not only the control of symptoms, but deep remission, which is related with a favourable prognosis. Thus, the determination of biological markers or biomarkers of intestinal inflammation play a crucial role. Many biomarkers have been extensively evaluated in IBD showing significant correlation with endoscopic lesions, risk of recurrence and response to treatment. One of the most important markers is faecal calprotectin (FC). Despite calprotectin limitations, this biomarker represents a reliable and noninvasive alternative to reduce the need for endoscopic procedures. FC has demonstrated its performance for regular monitoring of IBD patients, not only to the diagnosis for discriminating IBD from non-IBD diagnosis, but for assessing disease activity, relapse prediction and response to therapy. Although, FC provides better results than other biomarkers such as C-reactive protein and erythrocyte sedimentation rate, these surrogate markers of intestinal inflammation should not be used isolation but in combination with other clinical, endoscopic, radiological or/and histological parameters enabling a comprehensive assessment of IBD patients.

Key words: Faecal calprotectin; Inflammatory bowel disease; Biomarkers; Ulcerative colitis; Crohn's disease; Relapse

Core tip: The surveillance of inflammatory bowel disease (IBD) course is needed to select the patients with worse prognosis and to adapt an early therapeutic strategy. Faecal calprotectin constitutes a surrogate marker of intestinal inflammation and a robust alternative to invasive procedures as endoscopy. This biomarker has been demonstrated reliable and accuracy in different aspects of IBD such as diagnosis of IBD, activity

assessment, response to treatment and relapse prediction. Although a cut-off level of calprotectin has not been fully established, the combination with other biomarkers allows an appropriate management of the patient.

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INTRODUCTION

The main forms of inflammatory bowel disease (IBD) are the ulcerative colitis (UC) and Crohn's disease (CD). Both are chronic inflammatory disorders characterized by a relapsing-remitting clinical behavior. The course of IBD is unpredictable and can lead to cumulative intestinal tissue damage and complications which affect quality of life of patients[1]. The chronic nature of the disease requires a continuous assessment of activity to adapt the therapeutic strategy. Thus, physicians need reliable tools which allow to evaluate the disease activity and relapses risk.

Initially, the aim of therapy was to reach clinical remission, but this way is not enough to change the natural history of the disease. In recent years, the goal of treatment in IBD has changed and it is guided towards the mucosal healing, considered as a good predictor of the disease course, and associated with better patient outcomes[2,3].

Diagnosis and monitoring of IBD activity is based on a combination of clinical assessment, serologic and fecal markers of inflammation, cross-sectional imaging and endoscopy. Although endoscopy remains the gold standard for assessing IBD activity and mucosal healing, it has some risks and limitations: It is an invasive procedure, usually with low acceptance by the patient and potentially harmful, relatively high cost, it does not give information of the transmural inflammation, and finally is not well-known the timing of endoscopic evaluation. For this reason, numerous biomarkers have been proposed as surrogate markers of intestinal inflammation, and therefore also as potential markers of IBD activity. The biomarkers most extensively studied and commonly employed in clinical practice are C-reactive protein (CRP) and faecal calprotectin (FC).

This review offers a practical overview of the role of FC in several scenarios of clinical practice such as diagnosis of IBD, disease activity measurement, therapy response assessment and disease relapse prediction, describing its advantages and limitations (Table 1).

FAECAL CALPROTECTIN

Calprotectin is a calcium and zinc-binding protein which constitutes 60% of neutrophil cytosolic proteins[4,5], and that has functions such as antibacterial activity and induction of apoptosis[6]. Granulocytes produce FC at the site of mucosal inflammation increasing levels of this protein in faeces[7].

The FC level is a marker more specific of mucosal inflammation than CRP or erythrocyte sedimentation rate (ESR), which is less influenced by other non-intestinal conditions[8]. FC determination can be performed by enzyme-linked immunosorbent assay[5], and shows great stability at room temperature for a week[7]. This easy and inexpensive determination becomes calprotectin in a useful tool for monitoring of IBD patients.

Calprotectin presents some limitations in clinical practice. FC concentrations can be increase in non-IBD disorders; a cut-off level has not been well-established, and some authors described significant variability in a same patient[9]. Although a concentration < 50 μg/g may be considered upper limit of normal[10], an optimal cut-off for distinguishing IBD from other entities has not been fully described. The cut-off level of FC most commonly used varies from 50 to 200 μg/g[11]. von Roon *et al*[12] evaluated the diagnostic accuracy of FC for IBD and demonstrated that a cut-off level of 100 μg/g had better accuracy than 50 μg/g. Even, others authors increased the cut-off up to 150 μg/g[13].

The role of faecal calprotectin in the diagnosis of IBD

The diagnosis of IBD is based not only on clinical data, because symptoms are unspecific and present in other organic or functional disorders, but also, endoscopic, radiological and histological criteria are needed to confirm or exclude the diagnosis. The use of biological markers capable to differentiate between organic and functional diseases, would select those patients with suspected IBD which needs further invasive procedures such as colonoscopy. The role of biomarkers in this setting is variable.

FC has a great diagnostic accuracy for discriminating IBD from non-organic entities like has been reported in the literature[14] and evaluated in multiple studies[15-19].

Gisbert *et al*[14] reported an overall sensitivity of 80% and specificity of 76% for the diagnosis of IBD, reaching a higher accuracy for CD (sensitivity 83%, specificity 85%) than for UC (sensitivity 72%; specificity 74%). In a meta-analysis, von Roon *et al*[12] assessed the diagnostic precision of FC for IBD, and showed higher FC levels than non-IBD patients with a sensitivity of 95% and a specificity of 91%. Similar results have been published by other meta-analysis which included adult and pediatric studies with patients suspected to have IBD, with sensitivity and specificity of FC for distinction between IBD and irritable bowel syndrome of 93% and 96%, respectively. In pediatric population, this accuracy is lower reaching a sensitivity of FC of 0.92 (95%CI: 0.84-0.96) and specificity slightly lower 0.76 (95%CI: 0.62-0.86), probably due to the higher FC levels in healthy children up to 9 years of age[20].

This diagnostic accuracy of FC would decrease the

Table 1 Monitoring of inflammatory bowel disease with biomarkers

Advantages	Disadvantages
Relatively good acceptance	Not always well accepted by patients (faecal samples)
Non-invasive	Subject to non-specific variations
Relatively low cost	Predictive threshold values not fully established
May be combined to improve prediction	Imperfect correlation with mucosal healing and transmural healing
Can be repeated as a longitudinal monitoring tool	
Predictive value for	
Disease relapse	
Response to anti-TNF therapy	
Mucosal healing	

TNF: Tumor necrosis factor.

numbers of endoscopies needed up to 3-fold in adults and 35% in children[21] and, therefore, significantly reduces costs[22].

Therefore, FC is a reliable marker for organic gastrointestinal disorders, however, it is not specific for IBD, and other process can increase it such as neoplasms (colorectal cancer, polyps), gastrointestinal infections, other inflammatory entities (microscopic colitis, diverticulitis) and NSAID-induced enterocolitis[21]. A high value of FC constitutes a solid reason for performing a colonoscopy and confirming the diagnosis.

Although there is no established cut-off level to predict IBD, it is widely accepted that 50 μg/g is an accurate FC level to exclude organic intestinal disease with a high negative predictive value (NPV)[23]. Higher levels are not recommended because they would result in more false negative results and in this setting, the predictive negative value needs to be high in order to prevent delays in diagnosis. A normal value of FC makes unlikely the diagnosis of intestinal organic disease. The performance of FC with a cutoff of 50 μg/g as the first step to exclude organic disease seems reasonable, if the suspicion of IBD is not too high.

The diagnostic accuracy of FC for the diagnosis of IBD has been shown higher than other biomarkers such as CRP, ESR, anti-neutrophil cytoplasmic antibodies and anti-saccharomyces cerevisiae antibodies.

The role of faecal calprotectin in the monitoring of IBD

The role of faecal calprotectin to evaluate disease activity: The identification of inflammatory activity in a symptomatic IBD patient is crucial before changing the therapeutic strategy. Most of clinical indices employed to assess disease activity in IBD are based on patient symptoms and, therefore, subjective and poorly correlated with mucosal inflammation. The availability of biomarkers with a good correlation with clinical, endoscopic and histological activity is of capital relevance in daily clinical practice avoiding repeating invasive procedures. Moreover, fecal biomarkers are cheaper and easier, providing an important alternative to endoscopic procedures.

FC levels have shown a good correlation with the degree of inflammatory activity in IBD[24-26]. In CD, the median Pearson r correlation between the CD Endoscopic Index of Severity (CDEIS) and FC was 0.49, and for lactoferrin was 0.77[27-29]; the correlation with the simple endoscopic score for CD was similar for both FC (0.53) and lactoferrin (0.62)[27,30]. A meta-analysis with 550 patients evaluated the accuracy of CRP, FC and endoscopic scores, and it showed that in symptomatic patients (CDAI > 220), the sensitivity and specificity of CRP $\leqslant$ 5 mg/L or FC $\leqslant$ 200 μg/g to anticipate a CDEIS $\leqslant$ 6 was 83% and 71%, respectively[31].

In UC, FC levels show a better association with disease activity than in CD, and its correlation with endoscopic May score[28,32], Rachmilevitz index, and modified Baron score[33] was 0.72 (0.49-0.83).

Although no cut-off level has been validated, a FC > 200-250 μg/g has shown to have good accuracy in predicting endoscopic activity[28]. However, in CD with exclusively small bowel location, the sensitivity of FC to detect endoscopic lesions might be lower[34].

CRP has shown a sensitivity and specificity lower than FC for endoscopic disease activity both in UC and CD, so FC constitutes a more valuable marker than CRP in this context. In an appropriate scenario, the performance of FC could prevent the need for colonoscopy to confirm or exclude endoscopy activity in a symptomatic patient.

The role of faecal calprotectin to confirm mucosal healing and predict disease relapse: The course of IBD varies over time and while some patients have a favourable course with long periods of remission, others have a more aggressive disease, with unpredictable activity flare-up. Predicting the course along with the risk of relapse is useful because would allow to clinicians to individualize the management of each patient, conducting a more personalized approach and optimizing therapeutic strategies, minimizing adverse effects. The prediction of relapses would allow an early and intensive treatment in patients with worse prognosis. Studies examining this issue prospectively are limited and with inconclusive results regarding the frequency of determination of these biomarkers.

The capability to predict IBD relapse is one of the potential of the FC[25,35]. High levels of calprotectin in remission are associated with an increased risk of clinical relapse, with a sensitivity of 90% and specificity of 83%[18]. So, patients in clinical remission with high

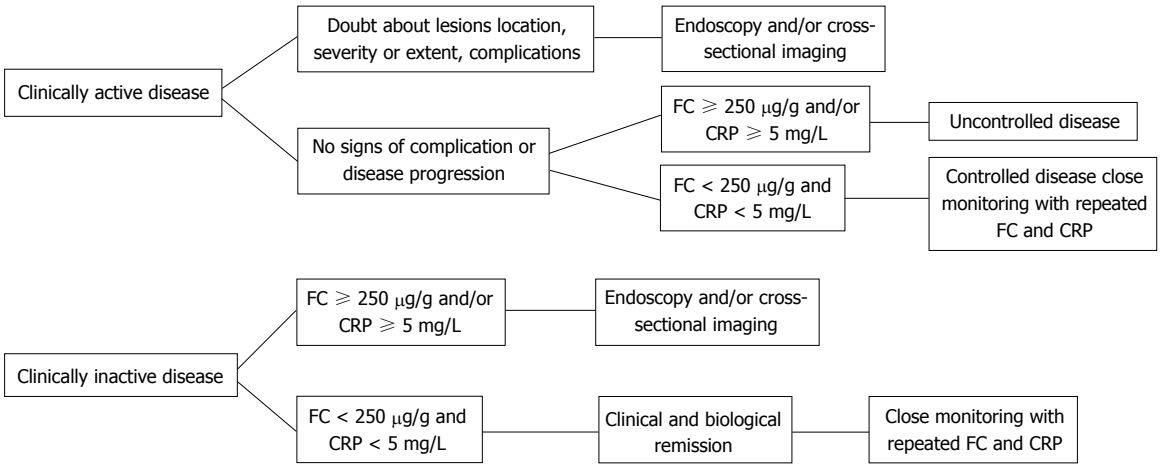

Figure 1 Algorithm for inflammatory bowel disease monitoring. A combination of clinical symptoms and biomarkers such as FC and CRP allow an individualized approach and a selection of patients for performing other invasive procedures and targeting treatment. FC: Faecalcalprotectin; CRP: C-reactive protein.

concentrations of FC had a risk of relapse of 2 and 14 times higher in CD and UC, respectively, compared to patients without elevated calprotectin[36]. However, CRP and ESR are not as helpful to predict disease's relapse, probably because theses biomarkers estimate intestinal inflammation indirectly.

It is necessary to clarify the predictive value of FC in UC and CD, its chronological relationship with the occurrence of relapse and the best cut-off point to determine relapse risk. D'Haens *et al*[28] showed that CD patients with a level of FC > 250 μg/g predicted the presence of large ulcers with a sensitivity of 60% and specificity of 80%, while a concentration of < 250 μg/g predicted mucosal healing (CDEIS < 3) with a sensitivity and specificity of 94% and 62%, respectively. A recent subanalysis of STORI study[37] suggested that the combination of FC (with threshold < 250 μg/g) and PCR (with threshold < 5 mg/L) can improve the capacity to predict mucosal healing with reasonably good sensitivity and specificity, around 70%. When considering inactive CD patients (CDAI ⩽ 150), the association of a PCR ⩽ 10 mg/L and a calprotectin ⩽ 200 μg/g has a sensitivity of 78% and a specificity of 58% for predicting no significant endoscopic activity (CDEIS ⩽ 3), with a positive predictive value between 65%-88% and 40%-70% NPV. So, if a colonoscopy is performed to 100 patients with CD in clinical remission with both biomarkers below this threshold, 30-40 colonoscopies could have been avoided. Patients with higher calprotectin or CRP levels should be considered holders of active intestinal lesions (Figure 1).

García-Sánchez *et al*[35] showed that the predictive value of FC was similar in UC and CD with colon involvement, and considering FC > 120 μg/g as a predictor of relapse risk with a sensitivity of 80% and a specificity of 60%. This predictive value is lower in patients with ileal CD. Although the appropriate frequency of determination of these markers is not well-established to date, data from the GETAID-STORI cohort indicates that both CRP and FC begin to increase their concentrations 4-6 mo before clinical relapse, so determinations every 3-4 mo should be sufficient to detect high levels and allow to clinicians to tailor therapeutic strategies[38].

The FC determination could also be useful to assess diseases activity evolution. Thus, Casellas *et al*[39] studied patients with clinically quiescent UC for 1 year or until clinical relapse; and they observed that FC values remains stable in patients with inactive UC, and increased in relapsing patients.

Ho *et al*[40] reported as FC levels could predict the colectomy risk in patients with acute severe UC. They evaluated 90 patients hospitalized for acute severe colitis and showed as very high levels of FC on admission were associated with an increased risk of colectomy. An initial calprotectin over 1900 μg/g predicted colectomy of 87% patients in the first year.

The role of faecal calprotectin to evaluate response to treatment: Another feature of faecal biomarkers is the rapid confirmation of drug efficacy after initiation of therapy. Usually, the evaluation of the response to treatment is based on clinical assessment, while endoscopy is rarely performed. It would be of great interest to have markers that reliably estimate the probability of response to different therapies. Thus, it is possible to identify subgroups of patients who would benefit from a particular therapeutic strategy as well as patients will have a poor response to the treatment being able to avoid exposure to them and the risk of adverse events. The lack of response to treatment may affect the quality of life of patients and increase their mortality.

Nowadays, the goal of treatment in IBD is to achieve mucosal healing, which has been associated with better outcomes and fewer relapses. However, to confirm absence of endoscopic lesions would be needed repeated endoscopic procedures. Therefore, biomarkers able to indirectly estimate this healing are imperative.

FC has been suggested as surrogate faecal marker of response to therapy. Several studies have demonstrated that normalization of calprotectin levels in

IBD patients after medical treatment is a marker that predicts the endoscopic healing. Decreased levels of FC after therapy are associated with clinical, endoscopic and histological improvement[41]. The normalization of calprotectin (< 50 mg/g) is more difficult to reach than the CRP normalization, so a significant decrease of FC could represent a deeper remission and a higher tissue healing[42,43].

When a steroid-free remission is achieved, de-escalation therapy may be tried to optimize benefit/risk. The combination of CRP and FC represents a good option to predict the risk of relapse after infliximab withdrawal[43].

For de-escalation of any drug or cessation of corticosteroids or mesalamine, a confirmation of biological remission with biomarkers such as CRP or FC can be sufficient. However, if we are willing to stop immunosuppressants or anti-TNF drugs, a confirmation of mucosal healing by endoscopy seems desirable[44].

The role of faecal calprotectin in postoperative recurrence assessment: There are scarce and conflicting data regarding the value of biomarkers in the postoperative setting to predict disease recurrence. FC usually returns to normal level by 2 mo postoperatively and any increase of its concentrations are associated with inflammatory recurrence[45].

Lobatón *et al*[34] suggested that FC is a more accurate and better surrogate marker of endoscopy activity in recurrent CD than clinical or serological markers, allowing to distinguish between postoperative recurrence patients (Rutgeert's score 2-4) and patients without recurrence (Rutgeert's score 0-1). In this study, using a cut-off value of FC of 203 μg/g reached a sensitivity of 75% and a specificity of 72%.

Beltrán *et al*[46] reported that FC is a useful early noninvasive marker for assessing recurrence of CD. A cut-off of 175 μg/g for FC is proposed.

CONCLUSION

The availability of biomarkers as FC represents a complementary tool to the clinical, endoscopic, radiological and histological procedures in the management of IBD patients. This surrogate marker is non-invasive, objective and non-expensive, and has a high accuracy for assessing different scenarios in IBD (to distinguish organic and functional disease, to evaluate disease activity, to predict risk of relapse, response to treatment and postoperative recurrence risk). FC can help to clinicians to avoid repeating invasive techniques selecting patients and to guide therapeutic decision. FC could be determined during follow-up allowing an early detection rather than just prediction of relapses. A combination of serological and faecal markers and endoscopy allow to the overall understanding of intestinal inflammation.

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Risk factors for osteoporosis in inflammatory bowel disease patients

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Abstract

Inflammatory bowel disease (IBD) patients exhibit higher risk for bone loss than the general population. The chronic inflammation causes a reduction in bone mineral density (BMD), which leads to osteopenia and osteoporosis. This article reviewed each risk factor for osteoporosis in IBD patients. Inflammation is one of the factors that contribute to osteoporosis in IBD patients, and the main system that is involved in bone loss is likely RANK/RANKL/osteoprotegerin. Smoking is a risk factor for bone loss and fractures, and many mechanisms have been proposed to explain this loss. Body composition also interferes in bone metabolism and increasing muscle mass may positively affect BMD. IBD patients frequently use corticosteroids, which stimulates osteoclastogenesis. IBD patients are also associated with vitamin D deficiency, which contributes to bone loss. However, infliximab therapy is associated with improvements in bone metabolism, but it is not clear whether the effects are because of inflammation improvement or infliximab use. Ulcerative colitis patients with proctocolectomy and ileal pouches and Crohn's disease patients with ostomy are also at risk for bone loss, and these patients should be closely monitored.

Key words: Bone mineral density; Crohn's disease; Osteoporosis; Ulcerative colitis; Inflammatory bowel disease; Risk factors

Core tip: Inflammatory bowel disease (IBD) is associated with bone loss. Some factors reduce bone mineral density and lead to osteopenia and osteoporosis. The major complication in osteoporosis is the increased risk of fracture, which may impact quality of life. This article reviews each risk factor for osteoporosis in IBD patients, like chronic inflammation, smoking, body composition, corticosteroid use, vitamin D deficiency, surgery, and

the effect of infliximab therapy.

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INTRODUCTION

Inflammatory bowel disease (IBD), which is primarily comprised of ulcerative colitis (UC) and Crohn's disease (CD), is associated with various systemic complications, such as extra-intestinal manifestations (EIMs)[1]. These complications are found in approximately 40% of IBD patients. The most widely known EIMs are skin lesions (erythema nodosum and pyoderma gangrenosum), articular manifestations and liver diseases (primary sclerosing cholangitis and primary biliary cirrhosis)[2,3].

IBD patients exhibit a higher risk for bone loss than the general population. Chronic inflammation causes a reduction in bone mineral density (BMD), which leads to osteopenia and osteoporosis. Cross-sectional studies reported a highly variable prevalence of low BMD in IBD patients. The prevalence of osteopenia and osteoporosis varies significantly depending on the study population, location, and design, but it ranges from 22%-77% and 17%-41%, respectively[4].

The incidence of inflammatory bowel disease seems stable in Western countries, but this disease has become more prevalent in Eastern countries, including Asia and Eastern Europe[5]. Extra-intestinal manifestations are also present in IBD patients in Eastern countries. Some studies demonstrated that these patients are at risk for bone loss and osteoporosis[6].

Dual-energy X-ray absorptiometry (DXA) is the current gold standard technique for the measurement of bone mass. Measurements are generally obtained at the femoral neck and lumbar spine. DXA results are typically expressed as the number of standard deviations (SD) above or below the expected mean for individuals of the same age, ethnicity and gender (Z score) or the mean of peak bone mass in young adults (T score)[7-9]. The World Health Organization reported formulated diagnostic ranges for osteoporosis based on T scores. Osteoporosis and osteopenia are defined by a T score below -2.5, and between -1 and -2.5, respectively. These recommendations were derived from postmenopausal Caucasian females. Therefore, caution must be exercised when extrapolating these data to other groups[9,10]. The current guidelines recommend DXA screening in IBD patients with one or more of the following risk factors: History of vertebral fractures, postmenopausal, male > 50 years of age, chronic corticosteroid therapy, or hypogonadism[9-11].

The major complication of bone loss and osteoporosis is the increased risk of fracture, especially non-traumatic fractures[12,13]. Bernstein *et al*[14] demonstrated that the incidence of fracture in persons with IBD is 40% greater than the general population. Other authors also reported similar findings[15-17]. Whether differences between IBD type (CD or UC) and BMD exist are not known. A cross-sectional population based study by Jahnsen *et al*[18] found that CD patients exhibited significantly reduced BMD compared to UC patients and healthy controls. A cohort of 3141 IBD patients in Taiwan also demonstrated a higher risk of osteoporosis in CD patients than UC patients[6]. However, these results are not consistent with other reports.

Whether gender interferes with BMD in IBD patients is not known. Ardizzone *et al*[19] demonstrated that spine and femur BMD Z and T scores were significantly lower in men than women UC patients, but this difference was not demonstrated in CD patients. A case control cross-sectional study of 113 CD patients found that female patients exhibited significantly decreased BMD of the femoral neck and the trochanteric region, but BMD was not significantly different from healthy controls in men[20].

Other risk factors associated to IBD or the general population are also related to the loss of bone mass with older age, postmenopausal status, smoking, malnutrition, physical inactivity, corticosteroid use for more than three months and vitamin D deficiency[21].

This review describes the specific risk factors for osteoporosis in IBD patients.

INFLAMMATION

Many factors exert important effects on bone metabolism, but there is increasing evidence that inflammation per se contributes to osteoporosis in IBD. Some studies in patients with newly diagnosed IBD demonstrated a reduction of BMD, even without the use of medications, such as corticosteroids[22,23].

Several chronic inflammatory disorders are associated with osteoporosis and an increased number of fractures. Inflammation is characterized by the production of cytokines, which is associated with increased bone resorption and reduced bone formation. The main system involved in the development of osteoporosis in IBD and other inflammatory diseases is likely the RANK/RANKL/osteoprotegerin[24].

The receptor activator of nuclear factor-B (RANK) is a transmembrane protein that is expressed on the surface of cells of hematopoietic origin that belongs to the TNF receptor family. RANK is the primary cytokine receptor in the development of osteoclastogenesis[25-28]. The ligand for RANK receptor (RANKL) is expressed on the surface of osteoblasts, mesenchymal cells and other cells, such as T and B lymphocytes. The binding of RANKL to RANK induces the differentiation of osteoclast precursors. RANKL also increases the resorptive activity of osteoclasts and prolongs their survival by suppressing

apoptosis[25-28].

Osteoblasts produce osteoprotegerin (OPG) as a control to maintain balance. OPG is a decoy receptor molecule that naturally binds RANKL to inhibit osteoclast activation and protect against bone loss[25-28]. Chronic inflammatory states mediated by T cell-produced cytokines affect osteoblasts and osteoclasts. Activated T cells produce RANKL and its soluble form, which directly triggers bone loss *via* the induction and activation of osteoclasts by RANK[29].

Several pro-inflammatory cytokines are involved in the activation of osteoclasts, such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), IL-6, IL-11, IL-15 and IL-17[30]. IL-6 plays an important role in the mediation of inflammatory osteoporosis, and it may also be involved in the pathways that lead to osteoporosis but are not elicited by inflammation[31].

Turk *et al*[32] demonstrated that patients with newly diagnosed and untreated CD exhibited elevated proinflammatory cytokines levels (IL-6, TNF-α, and IL-1) and increased free RANKL and OPG activity. These authors also observed a positive correlation between TNF-α and sRANK.

Disease activity interferes with bone metabolism. Some studies demonstrated that patients with disease in remission exhibit an increase in BMD. Reffitt *et al*[33] analyzed 137 IBD patients and demonstrated that patients with longer disease remission exhibited higher BMD.

SMOKING

Smoking has been recognized as a risk factor for bone loss and fractures for many years. Several mechanisms were proposed to explain the differences in BMD between smokers and non-smokers. However, the pathophysiological mechanisms underlying osteoporosis in cigarette smokers have not been fully explored[34,35].

Some studies demonstrated that the dose and duration of smoking may influence the effect of smoking on bone[35,36]. Smoking seems related to a vitamin D deficiency, and one possible explanation is that smoking alters the hepatic metabolism of vitamin D by influencing 25 hydroxylase in the liver, which lowers serum 25-hydroxyvitamin D[34,35]. There is also evidence that smoking alters gastrointestinal calcium absorption. Smokers lead an unhealthy lifestyle that includes low calcium/vitamin D intake, lack of exercise and alcohol ingestion, which affects bone health[34,35].

Whether smoking is associated with estradiol levels is controversial, but some studies demonstrated that smoking alters estrogen production and metabolism. There are some possible mechanisms. Nicotine may reduce estrogen production, and smoking enhances the hepatic metabolism of estradiol. Smokers exhibit higher serum sex-hormone binding globulin levels, which reduces free estradiol concentrations[34,35].

Few studies investigated the relationship between smoking and the RANK-RANKL-OPG system. Some reports demonstrated that smokers exhibit lower OPG levels without a difference in RANKL levels[36]. A cross-sectional study of 126 UC and 39 CD Iranian IBD patients demonstrated that femoral neck T scores were predicted by age, body mass index (BMI), smoking, and corticosteroid use. However, the association between smoking and BMD was not observed in the lumbar spine in this study[37]. Silvennoinen *et al*[38] evaluated the effect of smoking on BMD in 152 IBD patients (67 UC, 78 CD, 7 indeterminate colitis) and 73 controls and found that female IBD patients who currently smoked or with a previous history of smoking exhibited lower Z scores for the lumbar spine and femoral neck than female patients who had never smoked.

Smoking is also associated with relapses and disease activity (especially CD) and the need for steroids, which also negatively interferes with bone metabolism. The suspension of smoking is associated with more flare-ups in UC patients. However, smoking cessation should be encouraged in all IBD patients because it reduces other complications, such as cardiovascular disease, lung cancer and changes in bone health[39].

BODY COMPOSITION

Low BMI is a well-documented risk factor for low BMD and fracture[40]. Azzopardi *et al*[41] analyzed the risk factors for osteoporosis in 83 CD patients and found a significant association between BMI and BMD.

Many others studies also identified a positive association between BMD and BMI[42-44]. Atreja *et al*[45] also considered BMI a strong risk factor for altered bone metabolism and a way to identify osteoporotic patients who are missed by current guidelines. Leslie *et al*[46] studied 388 IBD patients and found that greater weight, height, and body mass measurements positively correlated with bone density at all sites. Fat and lean tissues exhibited positive relationships with BMD in this study, but lean tissue exhibited a much stronger correlation than fat tissue, especially for the total hip.

Low BMI is a risk factor for fractures, but whether obesity is a protective factor is not clear because obesity increases the risk of some osteoporotic fractures[47,48]. Johansson *et al*[49] published a recent meta-analysis of the association of fracture risk and BMI in women and concluded that there is a slight increase in osteoporotic fracture risk with increasing BMI after adjustment for BMD. Therefore, body composition appears more important than BMI in bone metabolism.

Mechanical loading of the muscles that act on the bone produce an anabolic effect, which results in osteogenesis[50,51]. Many IBD patients have reduced muscle (lean mass) because of nutritional factors, a sedentary lifestyle or medications, and these factors may lead to a reduced bone mass that is secondary to the decrease in mechanical stimulation of the skeleton[52].

A Canadian study analyzed the bone mass (bone mineral content) and muscle mass (lean mass) of 65 CD patients. Multiple regression analysis demonstrated

that only total lean mass was independently associated with lumbar bone mineral content (BMC), BMC in both hips and total BMC[52]. Lee *et al*[53] demonstrated a similar effect in a cohort of 61 CD patients. This study found that lean mass and muscle strength, but not fat mass, significantly correlated with regional and whole body BMD, but lean mass was the only independent predictor of hip BMD after multiple regression analysis. These authors concluded that maintaining or increasing muscle mass may positively affect BMD and prevent the development of osteopenia and osteoporosis.

GLUCOCORTICOID USE

Glucocorticoids (GCs) are frequently used in the treatment of inflammatory conditions, such as rheumatoid arthritis, systemic lupus erythematous, asthma and IBD. GC exposure is common in IBD patients, and over 50% of patients are exposed to systemic GCs within 5 years of diagnosis, and 20% have used at least 3 g of prednisone in any 1-year period[54].

Many studies consistently identified systemic GC use as a risk factor for osteoporosis and bone mineral loss in IBD patients[54,55]. Abraham *et al*[56] studied 166 IBD patients and demonstrated that the risk of osteoporosis was twice as high in patients who used corticosteroids [OR = 2.4 (1.5-3.4), P = 0.001].

Osteoporosis attributed to GC exposure is the most common etiology of drug-induced osteoporosis. Approximately 50% of patients receiving chronic GC therapy will develop osteopenia and fractures, and 17% of these patients will develop fractures within the first year of GC therapy[57].

Some risk factors for the development of fractures after steroid exposure were identified: Age older than 65 years, cumulative steroid dose (high GC dose and duration of treatment > 3 mo), positive family history of osteoporosis, low calcium intake, female sex, low bodyweight (BMI < 24 kg/m^2) and low BMD[57,58].

The mechanism of this loss is not fully understood. GC exposure alters the balance between osteoclast and osteoblast activity in bone metabolism. One important mechanism for the effects of GC on bone is osteoblastic dysfunction. GC inhibits stem cell differentiation into osteoblasts and induces osteoblast apoptosis, which decreases the secretion of osteoid matrix and new bone formation[57,59].

GCs increase the expression of RANKL and decrease the expression of its soluble decoy receptor OPG in stromal and osteoblastic cells. These alterations caused a greater differentiation of precursors into osteoclasts, which increases their resorptive activity and enhances bone reabsorption. There is also evidence that GCs directly prolong the lifespan of mature osteoclasts[57,59,60]. The increase in RANKL is only transient. Therefore, the failure of bone formation, rather than increased bone resorption, is likely the main mechanism underlying glucocorticoid-associated bone loss[58].

GCs also exhibit a negative effect on sex hormones status because GCs reduce estrogen and testosterone production. This negative effect of oral GCs on gonadal function may increase bone resorption[52,54].

GCs reduce intestinal calcium absorption and inhibit calcium reabsorption in the kidney, which indirectly leads to a negative net calcium balance and stimulates an increase in parathyroid hormone. These changes further increase the number of osteoclasts and stimulate bone resorption[57,58].

ROLE OF VITAMIN D

The role of vitamin D in IBD was not investigated in recent years. This vitamin primarily increases serum calcium and phosphate levels and promotes bone mineralization.

Vitamin D is available in two forms: Vitamin D3 (cholecalciferol), which is produced in the skin by exposure to sun light and obtained from animal sources, and vitamin D2 (ergocalciferol), which is obtained from plant sources. Vitamin D is metabolized in the liver to 25-hydroxyvitamin D [25-(OH) D], which circulates in the blood plasma and is stored in fat tissue and muscles. Metabolites of vitamin D are transported bound to albumin binding protein or vitamin D. This protein regulates the effects of the metabolites in target organs[61].

A study of 49 healthy young men demonstrated that free and bioavailable 25-(OH) D positively correlated with BMD, which suggests a possible benefit of vitamin D supplementation during deficiencies[62].

Vitamin D exerts its biological effects through the vitamin D receptor[61]. Multiple tissues and immune cells express this receptor, and these cells contain the enzyme that converts vitamin D into its active metabolite. Therefore, vitamin D appears to influence the innate immune response by inhibiting the maturation of dendritic cells and IL-12 and the adaptive immune response by inhibiting the production of IFN-γ, IL-17 and IL-21[63,64].

Several studies demonstrated a high prevalence of vitamin D deficiency in IBD patients. Many factors are attributed to this deficiency, and some of these factors are common to the general population, such as low sun exposure, inadequate intake, inactivity and other factors related to inflammatory disease, such as terminal ileum resection and low absorption due to the inflammatory process[64]. Disease activity is also associated with low levels of vitamin D in CD and UC patients[65,66].

Vitamin D deficiency leads to reduced calcium and secondary hyperparathyroidism, which stimulates osteoclastogenesis, increases bone resorption, and results in osteopenia and osteoporosis[64].

IS ANTI-TNF A PROTECTIVE FACTOR?

Elevated TNF-α concentration may play a role in dysfunctional bone metabolism in IBD. TNF-α is a major factor in the inactivation of osteoclasts. This cytokine induces osteoclast differentiation, increases osteoclast

Table 1 Infliximab effects on bone mineral density in Crohn's disease

Ref.	Study design[1]	Participants number	Endpoints	Results
Miheller *et al*[69]	Prospective	29 CD patients	Determine the effects of IFX on bone metabolism in CD patients	IFX improves bone metabolism in CD independently from the behavior of the disease
Abreu *et al*[70]	Prospective	38 CD patients	Assess the ability of IFX to increase bone formation measured by markers of bone turnover in active CD patients	Treatment with IFX was associated with increased markers of bone formation
Franchimont *et al*[71]	Prospective	71 CD patients, 68 controls	Assess the evolution of markers of bone turnover after IFX treatment for active CD	IFX induces improvement in biochemical markers of bone turnover
Mauro *et al*[72]	Retrospective	15 CD patients, 30 controls	Assess whether treatment with IFX had a beneficial effect on lumbar bone mass	Treatment with IFX was associated with significant increases in lumbar bone area, BMC and BMD in CD patients
Pazianas *et al*[73]	Retrospective	61 CD patients	Evaluate the effects of IFX administration on BMD in CD patients	IFX may work in synergy with bisphosphonates to provide additional increases in BMD in CD patients

[1]All were cohort study. CD: Crohn disease; IFX: Infliximab; BMC: Bone mineral content; BMD: Bone mineral density.

bone resorption, and protects these cells against apoptosis, which sensitizes osteoblasts to apoptosis and diminishes bone formation[67].

Infliximab (IFX) is a monoclonal antibody that exhibits high affinity and specificity for TNF-α. Anti-TNF therapy is an important IBD treatment because it allows for remission induction, relapse prevention and a decrease in corticosteroid use[68]. Some studies demonstrated the benefits of IFX use on BMD (Table 1). However, the exact mechanism of action of this anti-TNF in bone metabolism is not clear.

Miheller *et al*[69] investigated the effects of IFX on bone metabolism by measuring biochemical parameters in 29 CD patients and found that IFX increased osteocalcin levels (marker of bone formation) and reduced beta-Cross Laps levels (marker of bone resorption).

Abreu *et al*[70] observed increased bone alkaline phosphatase (bone formation marker) in 38 CD patients treated with IFX and no significant change in the dose of N-telopeptide of type Ⅰ collagen (NTX-marker of bone resorption). Franchimont *et al*[71] also examined the evolution of biochemical markers of bone metabolism after the first treatment with IFX in 71 CD patients. The authors of this study detected a normalization of bone markers after 8 wk of IFX treatment, with a median increase in formation markers of 14%-51%, according to the marker, and an approximately 10% reduction in bone resorption[71].

A retrospective study by Mauro *et al*[72] in 15 CD patients treated with IFX demonstrated significant increases in BMC and BMD in the lumbar spine compared to the control group.

The benefit of using IFX in BMD was also demonstrated with its associated use with bisphosphonates, as noted by Pazianas *et al*[73] in a retrospective cohort. They studied 61 CD patients, and patients who used bisphosphonates plus IFX experienced a greater increase in BMD than patients who used only bisphosphonate (6.7%/year *vs* 4.46%/year, $P < 0.05$).

The mechanism of action of IFX on bone metabolism is not well established, but its benefits in BMD may occur *via* the alteration of bone markers, the reduction of GC utilization and the induction of clinical and endoscopic remission.

Adalimumab is a human monoclonal IgG1 antibody that is specific for human TNF. It is also used in the treatment of IBD and other inflammatory diseases, such as rheumatoid arthritis and spondyloarthritis[74]. Studies demonstrated benefits in BMD in some patients using this therapy. Durnez *et al*[75] studied 59 patients with spondylo arthropathy treated with anti-TNF (infliximab, adalimumab or etarnecept) during a follow up of 6.5 years and found an increase in BMD of 11.8% in the lumbar spine and 3.6% in the trochanter.

Wijbrandts *et al*[76] conducted a prospective, open-label study of 50 rheumatoid arthritis patients. They analyzed the mineral density of the lumbar spine and femoral neck before and 1 year after adalimumab treatment. The authors observed no significant changes in BMD in lumbar spine (0.3%) or femoral neck (0.3%) and concluded that therapy with this anti-TNF does not increase BMD, but it can stop bone loss.

Another study by Krieckaert *et al*[77] evaluated the effect of long-term adalimumab use on BMD of the lumbar spine, hip and hands of rheumatoid arthritis patients. A total of 184 patients were studied, and hip and lumbar spine BMD remained stable after 1 year of treatment, but BMD in the hands decreased significantly by 1.41%. The mean BMD change per year was -0.58% and 0.07% for hip and lumbar spine, respectively, after a mean follow-up of 4.0 years (overall *P* value of hip was < 0.0001 and spine was 0.67). The authors considered that the BMD changes were associated to disease activity.

However, there are currently no published data investigating the effect of adalimumab on bone metabolism in IBD patients. There are also no data with certolizumab pegol.

EFFECT OF SURGERY

Proctocolectomy with ileal pouch-anal anastomosis

(IPAA) is the procedure of choice for the treatment of most patients with refractory UC, UC with dysplasia and familial adenomatous polyposis[78-80]. Some studies demonstrated an increase in long-term BMD after total colectomy with IPAA[79]. This surgery may improve BMD in UC patients, possibly due to the discontinuation of corticosteroids, improvement in nutritional status and a decreased production of cytokines by the diseased colon[78,79].

However, it is unclear whether total colectomy with ileal pouch provides benefits or detriments to BMD. In a study of 327 UC patients who underwent this surgery, 32% had low BMD 4 years after surgery, which suggests that bone loss continues after colectomy[78].

Possible risk factors and mechanisms of bone loss are considered. An ileal pouch changes the anatomy and function of the small intestine by reducing the absorption of bile salts, which contributes to the reduced absorption of vitamin D. The stasis of stool in the ileum in UC patients with IPAA promotes bacterial overgrowth, which causes deconjugation of bile salts and leads to the malabsorption of vitamin D[79]. Another mechanism is inflammation of the ileal pouch, which increases inflammatory cytokines levels, such as IL-1, IL-6 and TNF-α, and stimulates osteoclast activity and promotes bone loss[79].

Navaneethan *et al*[81] also found a lower BMD in UC patients undergoing total proctocolectomy and ileal pouch compared to the control group (31.1% *vs* 15.1%, $P < 0.001$). They also found that BMD was already low before surgery in 13 patients, and 7 (53.8%) of these patients exhibited an increase in BMD after surgery. Some studies demonstrated a higher incidence of fractures in UC patients with IPAA, ranging from 7%-15%[78,81].

IBD patients, particularly CD, are at increased risk of surgery based on disease severity and duration. The most common surgery for CD patients involves removal of the terminal ileum, which is associated to a vitamin D deficiency and the consequent secondary hyperparathyroidism, which promotes bone mass reduction. However, the relationship of these factors with osteoporosis in CD is not well defined[54].

Gupta *et al*[82] analyzed 126 patients with ostomy, and 95% of these patients had CD and ileostomy. This study also demonstrated a high frequency of fractures (9.5%) in CD patients after ostomy, with significantly higher rates in patients with low BMD. IBD patients with ostomy and low BMD also exhibited low BMI. Fractures were also five times more frequent in IBD patients with ostomy and low BMD.

IBD patients with ostomy are at higher risk for bone loss, and these patients should be monitored closely, especially patients with risk factors, such as low BMI and a previous history of fractures.

CONCLUSION

IBD is associated with bone loss, and patients are at increased risk of developing fractures. Many risk factors are associated with reductions in BMD in this population, including inflammation, smoking, body composition, glucocorticoid use, vitamin D deficiency and surgery. Infliximab seems to increase BMD, but the exact mechanism is not well established. More studies are needed to analyze the effect of other anti-TNF therapies in BMD.

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Promising biological therapies for ulcerative colitis: A review of the literature

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Abstract

Ulcerative colitis (UC) is a chronic lifelong condition characterized by alternating flare-ups and remission. There is no single known unifying cause, and the pathogenesis is multifactorial, with genetics, environmental factors, microbiota, and the immune system all playing roles. Current treatment modalities for UC include 5-aminosalicylates, corticosteroids, immunosuppressants (including purine antimetabolites, cyclosporine, and tacrolimus), and surgery. Therapeutic goals for UC are evolving. Medical treatment aims to induce remission and prevent relapse of disease activity. Infliximab, an anti-tumor necrosis factor (TNF)-α monoclonal antibody, is the first biological agent for the treatment of UC. Over the last decade, infliximab and adalimumab (anti-TNF-α agents) have been used for moderate to severe UC, and have been shown to be effective in inducing and maintaining remission. Recent studies have indicated that golimumab (another anti-TNF-α agent), tofacitinib (a Janus kinase inhibitor), and vedolizumab and etrolizumab (integrin antagonists), achieved good clinical remission and response rates in UC. Recently, golimumab and vedolizumab have been approved for UC by the United States Food and Drug Administration. Vedolizumab may be used as a first-line alternative to anti-TNF-α therapy in patients with an inadequate response to corticosteroids and/or immunosuppressants. Here, we provide updated information on various biological agents in the treatment of UC.

Key words: Ulcerative colitis; Biological therapy; Anti-tumor necrosis factor α agents; Janus kinase inhibitor; Anti-integrin agents

Core tip: Ulcerative colitis (UC) is a chronic lifelong condition characterized by alternating flare-ups and remission. Current treatment modalities for UC include 5-aminosalicylates, corticosteroids, immunosuppressants (*e.g.*, cyclosporine, tacrolimus), and surgery. Medical

treatment aims to induce remission and prevent relapse of disease activity. Infliximab and adalimumab have been used for moderate to severe UC, and are effective in inducing and maintaining remission in UC. Recent studies have indicated that golimumab, tofacitinib, vedolizumab and etrolizumab achieved good clinical remission and response rates in UC. In this review, we provide updated information on various biological agents in the treatment of UC.

Akiho H, Yokoyama A, Abe S, Nakazono Y, Murakami M, Otsuka Y, Fukawa K, Esaki M, Niina Y, Ogino H. Promising biological therapies for ulcerative colitis: A review of the literature. *World J Gastrointest Pathophysiol* 2015; 6(4): 219-227 Available from: URL: http://www.wjgnet.com/2150-5330/full/v6/i4/219.htm DOI: http://dx.doi.org/10.4291/wjgp.v6.i4.219

INTRODUCTION

Ulcerative colitis (UC) is an inflammatory disorder of the gastrointestinal tract that affects the colon and rectum. The symptoms of UC are rectal bleeding, diarrhea, and abdominal pain. It is a chronic lifelong condition characterized by alternating flare-ups and remission. There is no single known unifying cause, and the pathogenesis is multifactorial, with genetics, environmental factors, microbiota, and immune system all playing roles[1,2].

Medical treatment aims to induce remission and prevent relapse of disease activity, thereby minimizing the impact on quality of life. Current treatment modalities for UC include 5-aminosalicylates, corticosteroids, immunosuppressants (including purine antimetabolites, cyclosporine, and tacrolimus), and surgery. Therapeutic goals for the treatment of UC are evolving. Over the past decade there has been increasing evidence in favor of more objective measures of biological disease activity, including biomarkers such as C-reactive protein, faecal calprotectin, and the histological resolution of active inflammation in UC[3,4].

Infliximab, an anti-tumor necrosis factor (TNF)-α monoclonal antibody, is the first biological agent to have received United States Food and Drug Administration (FDA) approval. Over the last decade, infliximab has been used for moderate to severe UC, and has been shown to be effective in inducing and maintaining remission in UC[5]. Recently the TNF-α antagonists adalimumab and golimumab have shown a significant effect on UC[6,7].

In 2014, integrin receptor antagonist vedolizumab was approved for UC by the United States FDA and European Commission. In this review, we provide updated information on various biological agents in the treatment of UC.

ANTI-TNF-α AGENTS

TNF has been known to play a pivotal role in the pathogenesis of inflammatory bowel disease (IBD)[8]. When released by active macrophages and T lymphocytes, TNF initiates multiple biological reactions like modulates immune cell function, drives adaptive immune responses, triggers epithelium apoptosis and breaks epithelial barrier[9,10]. Anti-TNF-α agents have changed the treatment paradigm in the management of patients with UC.

Infliximab

As the first monoclonal TNF antibody approved for human treatment, infliximab is a purified, recombinant DNA-derived chimeric human-mouse IgG monoclonal antibody and contains murine heavy and light chain variable regions, ligated to genomic human heavy and light chain constant regions[11,12]. Infliximab can quickly form stable complexes with the human soluble or the membrane form of TNF and terminate the biological activity and signals of TNF[13]. With a serum half-life of 9.5 d and still detectable in serum of IBD patients 8 wk after infusion treatment, infliximab provides a useful strategy to neutralize TNF and to inhibit immune responses of IBD[14]. Infliximab is administered intravenously, and has been found to be effective for the treatment of moderate to severe UC in clinical trials[5,15]. Two randomized, double-blind, placebo-controlled studies-the Active UC Trials 1 and 2 (ACT 1 and ACT 2, respectively)-evaluated the efficacy of infliximab for induction and maintenance therapy in adults with UC[5]. Clinical response was defined as a decrease from baseline in the total Mayo score of $\geqslant$ 3 points and $\geqslant$ 30%, with an accompanying decrease in the subscore for rectal bleeding of $\geqslant$ 1 point or an absolute subscore for rectal bleeding of 0 or 1. Clinical remission was defined as a total Mayo score of $\leqslant$ 2 points, with no individual subscore exceeding 1 point. In ACT 1, 69.4% of patients who received 5 mg infliximab and 61.5% of those who received 10 mg had a clinical response at week 8, as compared with 37.2% of those who received placebo ($P < 0.001$ for both comparisons with placebo). In ACT 2, 64.5% of patients who received 5 mg infliximab and 69.2% of those who received 10 mg had a clinical response at week 8, as compared with 29.3% of those who received placebo ($P < 0.001$ for both comparisons with placebo). In both studies, patients who received infliximab were more likely to have a clinical response at week 30 ($P \leqslant 0.002$ for all comparisons). In ACT 1, more patients who received 5 or 10 mg infliximab had a clinical response at week 54 (45.5% and 44.3%, respectively) than did those who received placebo[5]. The results of ACT 1 and ACT 2 showed that infliximab had superior clinical efficacy compared with placebo, both in induction and maintenance phases.

Adalimumab

Adalimumab is a complete human IgG1 anti-TNF-α monoclonal Ab that has been generated through repertoire cloning. It binds to the soluble and transmembrane forms of TNF-α with high affinity, thereby preventing

TNF-α from binding to its receptors. *In vitro* studies have also demonstrated its effect on the induction of cell lysis and apoptosis[16]. It is generally administered at a dose of 40 mg subcutaneously every 2 wk, or at higher doses administered once a week. It is indicated for use in rheumatoid arthritis, psoriasis, ankylosing spondylitis, and moderate to severe Crohn's disease. Adalimumab can be self-administered by patients at home. Two randomized, double-blind, placebo-controlled studies-UC long-term remission and maintenance with adalimumab 1 and 2 (ULTRA 1 and ULTRA 2, respectively)-evaluated the efficacy of adalimumab for induction and maintenance therapy in UC patients[6,17]. ULTRA 1 was an 8-wk clinical trial investigating the use of adalimumab as induction therapy in patients with moderate to severe UC despite conventional therapy[17]. In this trial, 576 patients were divided into 160/80 mg and 80/40 mg groups, based on the loading dose, and then compared with the placebo group. At the end of 8 wk, the clinical remission rate of patients receiving adalimumab was twice that of the placebo group (P = 0.031). There was no significant difference in remission rates between patients receiving adalimumab 80/40 mg and placebo (P = 0.833). In ULTRA 2, a 52-wk randomized controlled study investigating the use of adalimumab as maintenance therapy, 494 patients were divided into 160/80 mg adalimumab and placebo groups. Overall rates of clinical remission at week 8 were 16.5% on adalimumab and 9.3% on placebo (P = 0.019); corresponding values for week 52 were 17.3% and 8.5% (P = 0.004). Among anti-TNF-α-naïve patients, rates of remission at week 8 were 21.3% on adalimumab and 11% on placebo (P = 0.017); corresponding values for week 52 were 22% and 12.4% (P = 0.029). Among patients who had previously received anti-TNF-α agents, rates of remission at week 8 were 9.2% on adalimumab and 6.9% on placebo (P = 0.559); corresponding values for week 52 were 10.2% and 3% (P = 0.039). Importantly, on sub-analysis, it was observed that the anti-TNF-α-naïve group exhibited approximately two times higher clinical remission rates at week 8 and week 52, compared with the placebo group. Though it is not direct comparison, infliximab is more likely to induce a favorable clinical outcome than adalimumab. The dose of adalimumab trough level might not enough to induce remission and maintenance for UC. More date are needed for dose escalation of adalimumab.

Up to 4 years of data for adalimumab-treated patients from ULTRA 1 and 2, and the open-label extension ULTRA 3 have been presented[18]. A total of 600/1094 patients enrolled in ULTRA 1 or 2 were randomized to receive adalimumab and induced in the intent to treat analyses. Of these, 199 patients remained on adalimumab after 4 years follow-up. Rates of remission according to partial Mayo score, remission according to inflammatory bowel disease questionnaire score, mucosal healing, and corticosteroid discontinuation at week 208 were 24.7%, 26.3%, 27.7% (nonresponder imputation), and 59.2% (observed), respectively. Of the patients who were followed up in ULTRA 3 (588/1094), a total of 360 patients remained on adalimumab 3 years later. Remission according to partial Mayo score and mucosal healing after ULTRA 1 or 2 to year 3 of ULTRA 3 were maintained by 63.6% and 59.9% of patients, respectively (nonresponder imputation). Nonresponder imputation method is used for dichotomous ("yes or no") or categorical variables, if a subject drops out of a study, that subject is assumed to be a non-responder, regardless of whether or not the subject was responding to treatment at the time of dropout.

Golimumab

Golimumab is a fully human IgG1 monoclonal antibody that targets TNF-α. It is subcutaneously administered and approved for use in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis. The affinity of golimumab for soluble TNF-α was similar to that of etanercept and greater than those of infliximab and adalimumab (2.4-fold and 7.1-fold, respectively). A similar pattern was observed regarding golimumab neutralization of soluble TNF-α in the cytotoxicity and endothelial cell activation assays. The IC_{50} values for golimumab were comparable to those for etanercept and ranged from 2.5- to 5.7-fold lower than those for infliximab and adalimumab. These *in vitro* bioassays suggest that a lower serum concentration of golimumab, compared with infliximab or adalimumab, would provide similar pharmacological effects in patients[19]. Two large, double-blinded, randomized, controlled trials have been conducted-the Program of UC Research Studies Utilizing an Investigation Treatment, which was divided into Subcutaneous and Maintenance phases (PURSUIT-SC, PURSUIT-M, respectively)[7,20]. In PURSUIT-SC, 774 patients were randomized to receive golimumab at week 6. The clinical response and remission rates showed a significant change in both the golimumab 200/100 mg and 400/200 mg groups (P < 0.0001)[10]. In PURSUIT-M, 464 patients who had responded to golimumab induction therapy in PURSUIT-SC were randomized to receive placebo or golimumab 50/100 mg every 4 wk for 52 wk. Clinical response was maintained through week 54 in 47.0% of patients receiving 50 mg golimumab, 49.7% of patients receiving 100 mg golimumab, and 31.2% of patients receiving placebo (P = 0.010 and P < 0.001, respectively). At weeks 30 and 54, a higher percentage of patients who received 100 mg golimumab were in clinical remission and had mucosal healing (27.8% and 42.4%) than patients given placebo (15.6% and 26.6%; P= 0.004 and P = 0.002, respectively) or 50 mg golimumab (23.2% and 41.7%, respectively)[7]. Though PURSUIT-M had included only persons who responded to induction in its maintenance phase, golimumab is more likely to induce a favorable clinical outcome than adalimumab (Table 1).

Janus kinase inhibitor: Various cytokines and intracellular messengers play a key role in pathogenesis of UC. Tyrosine kinases, such as Janus kinase 1 (JAK1)

Table 1 Clinical trials evaluating the efficacy of anti-tumor necrosis factor α agents in ulcerative colitis patients

Drug	Trial	Study population	Protocol	Follow-up (wk)	Outcome
Infliximab	ACT 1 Rutgeerts *et al*[5]	121	5 mg/kg *iv* at 0, 2, 6, and every 8 wk	54	69.4% ($P < 0.001$) clinical response at week 8 45.5% ($P < 0.001$) clinical response at week 54 38.8% ($P < 0.001$) clinical remission at week 8 34.7% ($P = 0.001$) clinical remission at week 54
		122	10 mg/kg *iv* at 0, 2, 6, and every 8 wk	54	61.5% ($P < 0.001$) clinical response at week 8 44.3% ($P < 0.001$) clinical response at week 54 32.0% ($P = 0.002$) clinical remission at week 8 34.4% ($P = 0.001$) clinical remission at week 54
	ACT 2 Rutgeerts *et al*[5]	121	5 mg/kg *iv* at 0, 2, 6, and every 8 wk	30	64.5% ($P < 0.001$) clinical response at week 8 47.1% ($P < 0.001$) clinical response at week 30 33.9% ($P < 0.001$) clinical remission at week 8 25.6% ($P = 0.003$) clinical remission at week 30
		120	10 mg/kg *iv* at 0, 2, 6, and every 8 wk	30	69.2% ($P < 0.001$) clinical response at week 8 60.0% ($P < 0.001$) clinical response at week 30 27.5% ($P < 0.001$) clinical remission at week 8 35.8% ($P < 0.001$) clinical remission at week 30
Adalimumab	ULTRA1 Reinisch *et al*[17]	130	80/40 mg *sc* 80 mg at week 0, 40 mg at week 2, 4 and 6	8	51.5% clinical response at week 8 10.0% ($P = 0.833$) clinical remission at week 8
		130	160/80 mg *sc* 160 mg at week 0, 80 mg at week 2, 40 mg at week 4 and 6		54.6% clinical response at week 8 18.5% ($P = 0.031$) clinical remission at week 8
	ULTRA2 Sandborn *et al*[6]	248	160/80 mg *sc* 160 mg at week 0, 80 mg at week 2, and then 40 mg every other week	52	16.5% ($P = 0.019$) clinical remission at week 8 17.3% ($P = 0.004$) clinical remission at week 52
	ULTRA3 Colombel *et al*[18]	360	40 mg *sc* every other week	208	63.6% remission per partial Mayo score at week 208
Golimumab	PURSUIT-SC[20]	253	200/100 mg *sc* 2 wk apart	6	51.6% ($P < 0.0001$) clinical response at week 6 17.8% ($P < 0.0001$) clinical remission at week 6
		257	400/200 mg *sc* 2 wk apart	6	54.9% ($P < 0.0001$) clinical response at week 6 17.9% ($P < 0.0001$) clinical remission at week 6
	PURSUIT-M[7]	151	50 mg *sc* every 4 wk	54	47% ($P = 0.010$) clinical response at week 54 23.2% clinical remission at week 54
		151	100 mg *sc* every 4 wk	54	49.7% ($P < 0.001$) clinical response at week 54 27.8% ($P = 0.004$) clinical remission at week 54

Clinical response was defined as a decrease from baseline in the total Mayo score of ⩾ 3 points and ⩾ 30%, with an accompanying decrease in the subscore for rectal bleeding of ⩾ 1 point or an absolute subscore for rectal bleeding of 0 or 1. Clinical remission was defined as a total Mayo score of ⩽ 2 points, with no individual subscore exceeding 1 point. *iv*: Intravenously; *sc*: Subcutaneously; ACT: Active Ulcerative Colitis Trials; ULTRA: Ulcerative Colitis Long-term Remission and Maintenance with Adalimumab; PURSUIT-SC: The Program of Ulcerative Colitis Research Studies Utilizing an Investigation Treatment, which was divided into Subcutaneous phases; PURSUIT-M: The Program of Ulcerative Colitis Research Studies Utilizing an Investigation Treatment, which was divided into Maintenance phases.

and JAK3, are intracellular molecules for the signal transmission of interleukins.

Tofacitinib

Tofacitinib (CP-690,550) is an oral inhibitor of JAK 1, 2 and 3 (with *in vitro* functional specificity for JAK1 and JAK3 over JAK2), which is expected to block signaling involving gamma-chain-containing cytokines including interleukins 2, 4, 7, 9, 15 and 21. In a double-blind, placebo-controlled, phase 2 trial, it was evaluated the efficacy of tofacitinib in 194 adults with moderate to severe active UC. Patients were randomly assigned to receive tofacitinib at a dose of 0.5, 3, 10 or 15 mg or placebo twice daily for 8 wk[21]. The primary outcome, clinical response at 8 wk, occurred in 32%, 48%, 61% and 78% of patients receiving tofacitinib at a dose of 0.5 mg ($P = 0.39$), 3 mg ($P = 0.55$), 10 mg ($P = 0.10$), and 15 mg ($P < 0.001$), respectively, as compared with 42% of patients receiving placebo. Clinical remission at 8 wk occurred in 13%, 33%, 48% and 41% of patients receiving tofacitinib at a dose of 0.5 mg ($P = 0.76$), 3 mg ($P = 0.01$), 10 mg ($P < 0.001$), and 15 mg ($P < 0.001$), respectively, as compared with 10% of patients receiving placebo[21]. Though the study population is small, 15 mg of tofacitinib showed most superior clinical response rate in induction phase than the other biological agents for UC.

INTEGRIN ANTAGONISTS

The integrin inhibitors are currently under development and have shown promising results to date. This group of drugs targets the leukocyte adhesion and trafficking systems, thereby reducing inflammation.

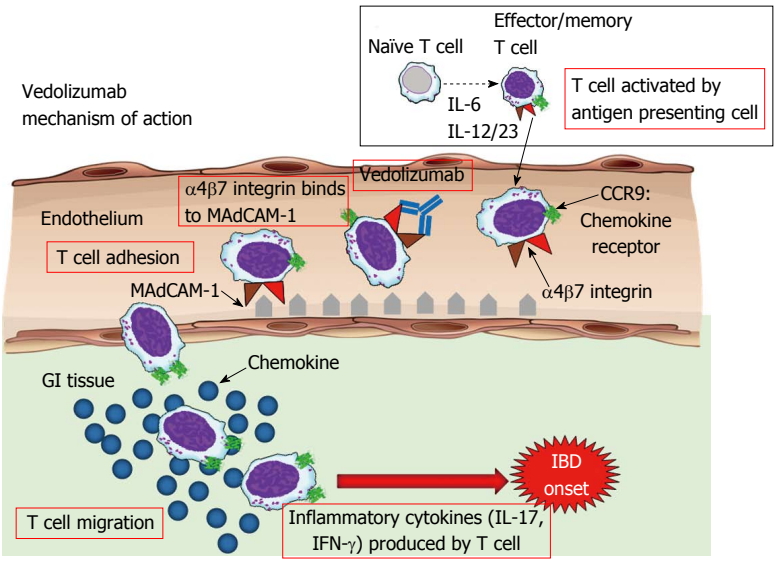

Figure 1 A mechanism of action that works to reduce inflammation in the gastrointestinal tract (Reprinted with permission from Takeda Pharmaceutical Co.). Vedolizumab selectively inhibits the movement of a discrete subset of T lymphocytes that preferentially migrate into inflamed GI tissue. Vedolizumab specifically binds to the α4β7 integrin, blocking its interaction with MAdCAM-1, which is mainly expressed on gut endothelial cells. This interaction facilitates lymphocyte homing to the gut and is an important contributor to inflammation that is a hallmark of ulcerative colitis. GI: Gastrointestinal; MAdCAM-1: Mucosal addressin cell adhesion molecule-1; IBD: Inflammatory bowel disease; CCR: Chemokine receptor; IL: Interleukine; IFN-γ: Interferon-γ.

Vedolizumab

The α4β7 integrin[22], a cell surface glycoprotein variably expressed on circulating B and T lymphocytes, interacts with mucosal addressin-cell adhesion molecule 1 (MAdCAM-1)[23] on the intestinal vasculature[24,25]. Vedolizumab, a humanized monoclonal antibody that specifically recognizes the α4β7 heterodimer, selectively blocks gut lymphocyte trafficking without interfering with trafficking to the central nervous system[26-28] (Figure 1).

A predecessor molecule (MLN02) showed proof-of-concept in a phase 2 trial[29]. Natalizumab, a monoclonal antibody with efficacy in multiple sclerosis and Crohn's disease, inhibits both α4β1 and α4β7 integrins and is associated with progressive multifocal leukoencephalopathy; a serious brain infection. Natalizumab and vedolizumab differ in that natalizumab blocks lymphocyte trafficking to multiple organs, including the brain and gut[30,31].

Randomized, double-blinded, placebo-controlled trials of vedolizumab in patients with active UC have been conducted[32]. In a trial of induction therapy, 374 patients (Cohort 1) received vedolizumab (300 mg) or placebo intravenously at weeks 0 and 2, and 521 patients (Cohort 2) received open-label vedolizumab at weeks 0 and 2, with disease evaluation at week 6. In a trial of maintenance therapy, patients in either cohort who had a response to vedolizumab at week 6 were randomly assigned to continue receiving vedolizumab every 8 or 4 wk or to switch to placebo for up to 52 wk. Response rates at week 6 were 47.1% and 25.5% among patients in the vedolizumab and placebo groups, respectively (difference with adjustment for stratification factors, 21.7% points; 95%CI: 11.6-31.7; $P < 0.001$). At week 52, 41.8% of patients who continued to receive vedolizumab every 8 wk and 44.8% of patients who continued to receive vedolizumab every 4 wk were in clinical remission (Mayo Clinic score ⩽ 2 and no subscore > 1), as compared with 15.9% of patients who switched to placebo [adjusted difference, 26.1% points for vedolizumab every 8 wk *vs* placebo (95%CI: 14.9-37.2; $P < 0.001$) and 29.1% points for vedolizumab every 4 wk *vs* placebo (95%CI: 17.9-40.4; $P < 0.001$)]. The frequency of adverse events was similar between the vedolizumab and placebo groups.

A network meta-analysis showed that in patients with moderate to severe active UC naïve to biological therapy, vedolizumab has similar efficacy to the anti-TNF-α antibodies, infliximab, adalimumab, and golimumab for induction of response and remission, and for maintenance of response and remission, but only vedolizumab had an incidence of serious adverse events lower than that of placebo[33]. Thus, in UC, vedolizumab may be used as a first-line alternative to anti-TNF-α therapy in patients with an inadequate response to corticosteroids and/or immunosuppressants. Vedolizumab may also be used in patients with UC not responding to anti-TNF therapy (primary nonresponders and secondary loss of response), because the drug has shown efficacy for this particular subpopulation[32].

The United States FDA and European Commission approved vedolizumab (Entyvio) for treatment of adults with moderate to severe active UC or CD in 2014. Up to 2015, vedolizumab for UC is approved in the United States, European Union, Canada, Israel, Switzerland, Puerto Rico, and Bosnia and Herzegovina. A phase 3, multicenter, randomized, double-blinded, placebo-controlled, parallel-group study to examine the efficacy, safety, and pharmacokinetics of MLN0002 (vedolizumab) in induction and maintenance therapy in Japanese patients with moderate or severe active UC is ongoing.

Table 2 Clinical trials evaluating the efficacy of Janus kinase inhibitor and integrin antagonists in ulcerative colitis patients

Drug	Trial	Study population	Protocol	Follow-up (wk)	Outcome
Tofacitinib	Sandborn *et al*[21]	31	0.5 mg *po* twice daily	8	32% (P = 0.39) clinical response at week 8 13% (P = 0.76) clinical remission at week 8
		33	3 mg *po* twice daily	8	48% (P = 0.55) clinical response at week 8 33% (P = 0.01) clinical remission at week 8
		33	10 mg *po* twice daily	8	61% (P = 0.10) clinical response at week 8 48% (P < 0.001) clinical remission at week 8
		49	15 mg *po* twice daily	8	78% (P < 0.001) clinical response at week 8 41% (P < 0.001) clinical remission at week 8
Vedolizumab	GEMINI 1[32]	225	300 mg *iv* at weeks 0, 2 and 6	6	47.1% (P < 0.001) clinical response at week 6 16.9% (P = 0.00) clinical remission at week 6
		122	300 mg *iv* at week 0, 2, 6 and every 4 wk	52	44.8% (P < 0.001) clinical remission at week 52
		125	300 mg *iv* at week 0, 2, 6 and every 8 wk	52	41.8% (P < 0.001) clinical remission at week 52
Etrolizumab	Vermeire *et al*[35]	39	100 mg *sc* at week 0, 4 and 8	10	21% (P = 0.0040) clinical remission at week 10
		39	420 mg *sc* loading dose then 300 mg at week 2, 4, and 8	10	10% (P = 0.048) clinical remission at week 10

po: Perorally; *iv*: Intravenously; *sc*: Subcutaneously.

Etrolizumab

Etrolizumab is an IgG1 humanized monoclonal antibody that selectively binds the subunit of the α4β7 and the αEβ7 integrin heterodimers in the intestine. Etrolizumab antagonizes α4β7/MAdCAM-1-mediated leukocyte recruitment in the intestinal vasculature and αEβ7/E-cadherin interactions, which are believed to be involved in retention of α4β7 cells in the intraepithelial compartment and in the migration and function of retinoic acid-producing $CD103^+$ dendritic cells expressing β7. The safety and pharmacology of etrolizumab were evaluated in a randomized phase 1 study in patients with moderate to severe UC. In the single ascending-dose stage, etrolizumab up to 10 mg/kg intravenously or 3.0 mg/kg subcutaneously showed no dose-limiting toxicity[34]. In a subsequent phase 2 study, patients with moderate to severe active UC were treated with three monthly doses of etrolizumab at 100 mg, a loading dose of etrolizumab at 420 mg and then 300 mg, or placebo[35]. Clinical remission occurred at week 10 in 20.5% of patients in the etrolizumab 100 mg group (P = 0.004), 10.3% of patients in the etrolizumab 420 mg loading dose group (P = 0.048), and no patients in the placebo group. The study population is so small, more studies are needed to confirm these data (Table 2).

Safety: Recent studies have shown that a few patients experience adverse events with biological agents. For adverse events, such as infections, neoplasms are related to the immunosuppressive effects of biological agents. Patients who are administered biological agents frequently develop antibodies against these drugs. This problem is more frequent with chimeric agents like infliximab than fully humanized agents like adalimumab.

Infliximab: Infliximab is a chimeric monoclonal antibody with a protein sequence that is 75% human and 25% mouse; therefore, human antichimeric antibody formation can occur in the blood. The presence of human antichimeric antibody is associated with an increased risk of infusion reactions during administration and reduced clinical efficacy. The common adverse events of infliximab are acute infusion reaction, and infection such as reactivation of tuberculosis.

As with other immunomodulatory drugs, infliximab therapy increases the risk of developing non-serious infections (RR approximately equal to 2); however, the data on serious infections are inconsistent[36]. Examples of reported serious infections include sepsis, pneumonia, cellulitis and intra-abdominal abscess[37]. Thus, infliximab should not be administered to a patient who has a clinically active infection. Patients who are at a high risk of chronic hepatitis B infection should be screened before the initiation of infliximab therapy.

Approximately 10% of infliximab infusions are associated with mild reactions such as headache, dizziness, fever, chills, chest pain, cough dyspnea or pruritus. These reactions occur within 1-2 h after infusion and can be alleviated by reducing the rate of infusion or by pretreatment with an H1-receptor antagonist[36,37]. In the ACT 1 and ACT 2 trials, 11.4% of the patients receiving infliximab experienced infusion reactions (44 of 484), compared with 9.4% of those receiving a placebo (23 of 244)[5].

For reasons that are unclear, 1 in 1000 infliximab infusions results in a serious reaction[37]. Delayed hypersensitivity-like reactions (serum sickness-like disorders) can occur 3-14 d after episodic infliximab infusions and include, but are not limited to, myalgia, fever, rash, pruritus, dysphagia, urticaria and headache[37]. In the ACT 1 and ACT 2 trials, three patients who received either 5 or 10 mg/kg infliximab had delayed hypersensitivity reactions (n = 484), as compared with two patients in the placebo study group (n = 244)[5].

Cases of aplastic anemia, pancytopenia, vasculitis, hepatitis, reversible mono/polyneuropathy and demye-

lination have been attributed to infliximab therapy[38].

At present, there is no consensus regarding the estimated lymphoma risk for patients treated with infliximab[36]. However, most experts believe that immunosuppression does impart some small cumulative risk of malignancy. The development of hepatosplenic T-cell lymphoma, a rare malignancy, has been reported in pediatric patients receiving infliximab treatment for Crohn's disease in the United States[38,39].

Adalimumab: A total of 1010 patients received at least one dose of adalimumab in the ULTRA 1, 2 and 3 trials. The most frequently reported serious adverse event was worsening or flare of UC. Two serious events of cytomegalovirus colitis were reported. After the double-blind study period, one serious infection of tuberculosis and two treatment-emergent fatal adverse events were reported. Three events of B-cell lymphoma occurred during ULTRA 3. All three patients had a history of smoking and either previous or concomitant azathioprine use[18].

Golimumab: The most commonly observed adverse events in golimumab- and placebo-treated patients were headache and nasopharyngitis. Overall, the incidences of serious adverse events (3.0% *vs* 6.1%), including serious infections (0.5% *vs* 1.8%), were also similar, respectively, for golimumab- and placebo-treated patients. The most common serious adverse event was the exacerbation of UC, reported by eight (1.1%) golimumab-treated and eight (2.4%) placebo-treated patients. The only serious infection reported by more than one patient was pneumonia (one receiving 200/100 mg golimumab and one placebo patient). One patient (400/200 mg) died from peritonitis and sepsis after surgical complications related to an ischiorectal abscess and subsequent bowel perforation after surgery; this patient was receiving concomitant 20 mg prednisolone. One patient (400/200 mg) had a demyelinating disorder reported after the patient completed PURSUIT-SC induction and subsequently was randomized to placebo in the maintenance study. Two opportunistic infections were reported up to week 6: Esophageal candidiasis (400/200 mg golimumab) and cytomegalovirus infection (placebo). Neither event was reported as serious. No patient developed active tuberculosis[20].

Tofacitinib: The most commonly reported adverse events related to infection were influenza and nasopharyngitis (in six patients each). During the study period, the absolute neutrophil count was < 1500 cells/mm^3 in three patients receiving tofacitinib (one at a dose of 10 mg twice daily and two at a dose of 15 mg twice daily); it was < 1000 cells/mm^3 in none of the patients[21].

Vedolizumab: In the large GEMINI I study, no significant difference was observed among the study groups for the most commonly reported adverse events: Namely, flare of UC, headache, nasopharyngitis and arthralgia. Serious infections were no more common with vedolizumab than with placebo. No cases of progressive multifocal leukoencephalopathy occurred[32].

Etrolizumab: Patients in the 100 mg etrolizumab group had higher rates of rash, influenza-like illness, and arthralgia than did those in the placebo or 300 mg etrolizumab plus loading dose (LD) groups; all of these events were regarded as mild to moderate in severity. Serious adverse events were reported in 12 patients; five of these were related to UC (two in the 100 mg etrolizumab group; one in the 300 mg etrolizumab plus LD group; and two in the placebo group; Appendix)[35].

CONCLUSION

A number of biological agents are currently available for treatment of UC. These agents serve as another appropriate treatment option for gastrointestinal clinicians in patients with moderate to severe UC who may not be effectively treated with conventional agents. Various cytokines and intracellular messengers are involved in the pathogenesis of UC; thus, further discovery and development of new agents are required.

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ORIGINAL ARTICLE

Basic Study

Predictive factors at birth of the severity of gastroschisis

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Abstract

AIM: To establish children born with gastroschisis (GS).

METHODS: We performed a retrospective study covering the period from January 2000 to December 2007. The following variables were analyzed for each child: Weight, sex, apgar, perforations, atresia, volvulus, bowel lenght, subjective description of perivisceritis, duration of parenteral nutrition, first nasogastric milk feeding, total milk feeding, necrotizing enterocolitis, average period of hospitalization and mortality. For statistical analysis, descriptive data are reported as mean ± standard deviation and median (range). The non parametric test of Mann-Whitney was used. The threshold for statistical significance was $P < 0.05$ (Two-Tailed).

RESULTS: Sixty-eight cases of GS were studied. We found nine cases of perforations, eight of volvulus, 12 of atresia and 49 children with subjective description of perivisceritis (72%). The mortality rate was 12% (eight deaths). Average duration of total parenteral nutrition was 56.7 d (8-950; median: 22), with five cases of necrotizing enterocolitis. Average length of hospitalization for 60 of our patients was 54.7 d (2-370;

median: 25.5). The presence of intestinal atresia was the only factor correlated with prolonged parenteral nutrition, delayed total oral milk feeding and longer hospitalization.

CONCLUSION: In our study, intestinal atresia was our predictive factor of the severity of GS.

Key words: Gastroschisis; Perivisceritis; Bowel atresia; Volvulus

Core tip: Gastroschisis (GS) is defined as a full-thickness congenital abdominal wall defect usually situated on the right side of the umbilicus, with intestines protruding into the amniotic fluid without any protective membrane. The amniotic fluid creates an inflammation of the bowel wall, called perivisceritis. Associated with intestinal abnormalities are malrotation and a degree of short bowel: Volvulus, perforation and atresia may also be found. Our study shows that for babies born with GS, intestinal atresia is the only factor of prediction of the need for early and full enteral feeding, for its duration, and for the length of hospitalization.

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INTRODUCTION

Gastroschisis (GS) is defined as a full-thickness congenital abdominal wall defect usually situated on the right side of the umbilicus, with intestines protruding into the amniotic fluid without any protective membrane[1]. The amniotic fluid creates an inflammation of the bowel wall, called perivisceritis[2]. Associated with intestinal abnormalities are malrotation and a degree of short bowel: Volvulus, perforation and atresia may also be found[3]. The degree of inflammation of the bowel and the presence of intestinal abnormalities are supposed to reflect the severity of the malformation, determining the surgical procedure and affecting the clinical outcome[4]. The duration of total parenteral nutrition (TPN), the timing of the introduction of normal feeding, the average length of hospitalization and in some cases death is all dependent on the severity of the malformation.

A prenatal ultrasound (US) diagnosis makes it possible to inform and prepare the parents, even though it remains difficult to predict whether the affected children will have a high or a low risk of abdominal complications, and how long they will need to be hospitalized[5,6]. If the defect closes by itself before birth, ischemia of the bowel may provoke total bowel necrosis. The prenatal closure of the abdominal wall is therefore not always a good sign and may be associated with midgut infarction, short bowel or even vanishing midgut[7,8].

Surgical management starts with the clinical observation of the anatomical anomalies accompanying GS and the search for associated malformations. Primary or delayed repair of the abdominal wall is then discussed[9]. Primary repair entails returning the bowel into its cavity soon after birth with a surgical procedure. But this primary abdominal closure is not always possible and depends on the age, weight and clinical condition of the baby, as well as on the amount of viscera protruding from the abdominal cavity: If it is too important, the immediate closure of the abdominal wall may cause excessive pressure. Delayed repair entails the use of a suspended pouch, called a "silo", containing the externalized bowel loops, with a gradual return of the bowel into the abdominal cavity and the closure of the abdominal wall a few days later[10]. The "silo" reduces the risk of excessive pressure, hypothermia and dehydration.

Volvulus, perforation and atresia may require resection, anastomosis or the creation of a stoma. The presence of these associated intestinal anomalies influences surgical and post surgical clinical management, for instance regarding the decision to perform a stoma or delay oral feeding because of the risk of necrotizing enterocolitis (NEC).

We performed a retrospective review of all children born with GS in our hospital over an eight-year period. The aim of this study is to establish which parameters such as bowel volvulus and/or atresia may help to predict the duration of TPN before the initiation of milk intake and eventual total oral milk feeding, and also the length of hospitalization.

MATERIALS AND METHODS

We reviewed the files of all children admitted to our hospital with a diagnosis of GS from January 2000 to December 2007. Our records on the mothers indicate the duration of the pregnancy and the method of delivery. In our records on the children, we registered: weight, sex, apgar scores, acidosis at birth, length of intubation, bowel aspect, presence of necrosis, of perforation, of volvulus without necrosis, length of TPN, first milk feeding, total milk feeding, NEC, average length of hospitalization and, in some cases, time of death. Signed informed consent for this study was obtained from the appropriate local institutional Human Research Board.

Mothers were followed during the prenatal period by means of regular ultrasound exams. Bowel dilation, thickness of the abdominal wall, motility of the bowel, quantity of amniotic fluid, and fetal development were controlled during gestation. Vaginal delivery of the babies was proposed in our maternity department to avoid post-natal transfer. Scheduled vaginal provocation was planned, unless obstetric considerations led us to opt for caesarian delivery. When faced with fetal indications such

as worsening fetal status, progressive bowel dilation or loss of bowel movement, or with maternal problems, we performed an early delivery. Prenatal discussion sessions were organized between physicians of different specializations, and parents had the opportunity to meet with pediatric surgeons, neonatologists and geneticists.

The diagnosis of GS was confirmed immediately after delivery by neonatologists and pediatric surgeons. Primary surgery, if possible within the few hours following delivery, was our preferred mode of wall repair. After a rapid physical assessment, a nasogastric tube was placed in the stomach and intravenous fluid and antibiotics were given. The child was rapidly brought to the operating theater after routine resuscitation. The colon was irrigated with 5% n-acetyl cysteine diluted in warm normal saline solution to evacuate meconium. A Foley catheter was placed in the bladder for urinary drainage and for measurement of intra-abdominal pressure after closure of the abdominal wall.

Under total anaesthesia, the bowel loops were reduced gently into the abdominal cavity, fascia were separated from the skin and repaired with absorbable sutures. The abdominal wall was closed layer by layer. Urine production, absence of a compartment syndrome and good perfusion of both legs were controlled throughout. A broviac catheter was placed for TPN. The babies were then taken to the intensive care ward with intubation/ventilation, and given pain killers and drugs for wall relaxation for as long as necessary. Bladder pressure was measured continuously and maintained below 15 mmHg. Pulse oxymetry was measured on the feet. Nasogastric suction was maintained until bowel function returned. TPN was provided until adequate oral nutrition became possible.

Primary closure was not always indicated, and the decision not to close the abdominal wall was taken either during surgery, when the introduction of the bowel into the abdominal cavity induced a compartment syndrome, or if the baby could not be brought to the operating room because of his weight or the presence of associated malformations. In these cases, we used a protective "silo" to allow staged reduction of the bowel over a period of several days. After this period of progressive bowel reintegration into the abdominal cavity, the abdominal wall was closed layer by layer as described above.

Short bowel syndrome normally defines a functional state dependent on the degree to which the normal absorbtive capacity of the small intestine is compromised[11]. In our paper, short bowel is defined according to either the length of small intestine present after abdominal closure, or the length left after intestinal resections in cases of intestinal atresia. We defined two categories: more than 100 cm or less than 50 cm.

Inflammation of the bowel was defined subjectively at birth and during surgery on the basis of the aspect of the bowel wall, the presence of large amount of fibrin, the abnormal thickness of the bowel wall and the absence of bowel movement or contraction after stimulation. A less inflamed bowel was defined subjectively, on the basis of the presence of a small amount of fibrin, a practically normal appearance and thickness of the bowel wall, and bowel movement under stimulation.

Oral feeding was started as soon as possible through a gastric catheter. One milliliter per hour was given at the beginning, and this amount was gradually increased depending on the color and quantity of fluid aspiration, the abdominal distention and stools production. The amount needed for full feeding was determined by the weight of the baby.

For statistical analysis, descriptive data are reported as mean ± standard deviation and median (range). The non parametric test of Mann-Whitney was used as the distribution of different variables was not always regular. The threshold for statistical significance was $P < 0.05$ (Two-Tailed). All statistical analyses were performed by a biostatistician using the statistical software SAS for Windows (SAS release 8.2, 2002, Cupertino, California, United States).

RESULTS

From January 2000 to December 2007, 72 babies were deemed eligible for the study. But four were immediately excluded because their records were incomplete. We therefore retained 68 cases of GS ($n = 68$) for our study.

The mean age of the mothers was 23.1 years, with a range from 15 to 34 years. The mean delivery time was 35.6 wk (median: 36 wk), with vaginal delivery in 67% of cases. A diagnosis of intrauterine growth restriction was made in 22.4% ($n = 15$) of cases, and confirmed at birth in all cases. No intrauterine deaths were reported for fetuses with intrauterine growth restriction. Oligohydramnios was observed in 24% ($n = 16$) of mothers. No complications were reported during delivery. Average weight at birth was 2501 g and 53% were girls.

We found eight volvulus, 12 atresia, nine perforations and six stenosis, 39 bowels less than 100 cm and 22 less than 50 cm (Table 1). Subjective description has done by the surgeon of the presence of a perivisceritis were found in 49 children (72%). We used silos in 17 cases (25%), for an average period of 5.94 d. Average age at surgery was 2.45 d.

A post-natal mortality rate of 12% (8/68) was observed. Three of these babies (3/68) showed concomitant fetal abnormalities incompatible with life; three (3/68) died, respectively from short bowel syndrome requiring a bowel transplant, NEC with fistula, and after surgery for a chylothorax; and two more (2/68), who presented small bowel necroses requiring multiple surgery, also died shortly after birth.

Mean duration of TPN was 56.7 d (range: 8-950 d; median: 22 d) with five NEC; four newborns (6%) received TPN for less than ten days; 20 patients (29.9%) required TPN for 30 d or more, including nine patients ($n = 9$) who presented no other complications besides GS. Of those 20 patients, eight ($n = 8$) required multiple

Table 1 Summary of clinical conditions for the 68 cases of gastroschisis

	Mean	Median
Birth weight	2501 g	
Gestational age	35.6 wk	36 wk
Female sex	53%	
Mother's age	23 yr	
Timing of closure	2.5 d	
Silo	25% (17)	
Severely inflamed	72% (49)	
Perforations	13.2% (9)	
Volvulus	11.7% (8)	
Atresia	17.6% (12)	
Bowel less than 100 cm	66% (39)	
Short bowel less than 50 cm	30% (21)	
Stenosis	8.8% (6)	
NEC	7.35% (5)	
Mortality	11.7% (8)	
For 60 children (68 minus eight deaths)		
Intubation in days	8.5 d	5 d
1st feeding	17.3 d	11 d
Timing of total milk feeding		
Days of TPN	56.7 d	22 d
Hospitalization	54.7 d	25.5 d

NEC: Necrotizing enterocolitis; TPN: Total parenteral nutrition.

surgery, six had atresia (n = 6), three NEC (n = 3), and three eventually died (n = 3).

Mean duration of intubation was 8.47 d (median: 5 d). Mean duration of hospitalization was 54.7 d (2-370; median: 25.5) for our 60 cases of GS (Table 1). Twenty-six patients (23.9%) were hospitalized for 50 d or more, among whom nine required (n = 9) multiple surgery, eight were cases of atresia (n = 8), three of volvulus (n = 3), three of NEC (n = 3), and the three who eventually died (n = 3).

The median period of time before initiation of nasogastric milk, the median duration of TPN, the median period of time until the start of total oral milk intake and the median duration of hospitalization are summarized in Table 2.

There were a total of 12 cases with intestinal atresia and 48 without atresia. The median period of time before initiation of nasogastric milk, the median duration of TPN, the median period of time until the start of total oral milk intake and the median duration of hospitalization are summarized in Table 2.

There were eight cases of volvulus and 52 children without volvulus. The median period of time before initiation of nasogastric milk, the median duration of TPN, the median period of time until the start of total oral milk intake and the median duration of hospitalization are summarized in Table 2.

DISCUSSION

Our study shows that for babies born with GS, intestinal atresia is the only factor of prediction of the need for early and full enteral feeding, for its duration, and for the length of hospitalization.

The etiology of GS has not yet been ascertained, but low socioeconomic status, poor maternal education, drug abuse, in particular with cocaine, abuse of tobacco and alcohol, and young maternal age (less than 20 years old) are associated with GS[11-14]. Although the survival rate for babies born with GS has improved and is now practically 85%, short and long-term morbidity is still a serious problem. The mortality rate reported in recent literature varies greatly (2.4% to 11%)[15,16]. but the mortality rate reported in our study (11.7%) tallies with the values reported in most studies. Prematurity, intrauterine growth retardation and the presence of a congenital circulatory or pulmonary anomaly are external factors associated with a poorer outcome for children born with GS[3]. In our study, three babies (3/68) showed concomitant fetal abnormalities incompatible with life; finally, five babies died from GS and associated bowel complications (5/68, 7.3%).

Many authors have attempted to establish a prognosis of post-natal morbidity in cases of GS by studying various prenatal US findings, such as amniotic fluid volume, small bowel diameter, maximum bowel diameter, maximum thickness of the bowel wall, intrauterine growth restriction, Doppler velocimetry of the superior mesenteric artery, and the presence of other anomalies. Other studies have tried, based either on prenatal US or on bowel examination at birth, to establish a prognosis for the length of hospitalization, the duration of TPN or the timing of introduction of normal feeding. It appears, however, that antenatal screening cannot reliably predict morbidity, and the fact that no consensus emerges from the different studies is due mainly to the small sample size, but also to the difficulty in correlating imaging findings with clinical outcome. Some studies found a significant association between intra-abdominal bowel dilation and bowel atresia[5,17,18]. Japaraj *et al*[6] found that the occurrence of polyhydramnios was significantly associated with a higher rate of severe bowel complications such as atresia, perforation and necrosis. Intestinal atresia has been described as a significant risk factor of morbidity and mortality, due to the fact that the dilated bowel causes increased abdominal pressure during and after abdominal closure[15]. While, in both our groups (with atresia and without atresia), nasogastric milk feeding was initiated after an equal number of days following surgery (11 d and 10 d respectively), the median duration of TPN was clearly and significantly greater in the "atresia group", and the period of time before total milk feeding could be introduced was longer. The mortality rate in the "atresia group" was not higher, even though, in 12 cases, atresia was associated with three perforations and intestinal necrosis which required several surgical procedures. In one case, atresia was also associated with a chronic intestinal pseudo obstruction. The higher morbidity in this group also influenced the duration of hospitalization, which was definitely longer, due to surgical complications, prolonged TPN and delayed total milk feeding.

Short-term and long-term outcomes of GS are

Table 2 Comparison in days between two groups of 60 children depending on the presence of atresia, volvulus and perivisceritis (68 minus eight deaths)

	Atresia (n = 12)	Non atresia (n = 48)	Mann-Whitney test	Volvulus (n = 8)	No volvulus (n = 52)	Mann-Whitney test	Subjective perivisceritis (n = 49)	Subjective low perivisceritis (n = 19)	Mann-Whitney test
TPN duration	60	20	0.005	36	20	0.140	34.5	22	0.564
Start of total oral milk intake	10	11	0.809	11	7.5	0.212	11	10.5	0.569
Total oral milk intake	56	20	0.05	22	17	0.468	36	21	0.196
Mean hospitalization	64	24.5	0.021	48	25.5	0.309	39	25.5	0.505

TPN: Total parenteral nutrition.

well-known but difficult to predict. Many studies have demonstrated that the presence of a compromised bowel is associated with a significant increase in the number of surgical procedures, a longer period of full enteral feeding and a prolonged hospitalization[15,19]. Prolonged TPN, with its risk of sepsis, is directly related to intestinal recuperation, and the morbidity of GS is closely related to intestinal damage[20]. While gastrointestinal complications such as matting between the loops, malrotation, volvulus, perforations and atresia increase the complexity of early management[19,21], later management may be complicated by the presence of problems of absorption, intestinal dysmotility, obstruction, NEC, infarction and stenosis[1,3,9].

The pathogenesis of secondary bowel lesions is not fully understood, but both chemical and mechanical origins are concerned[2]. We know that prolonged contact with the amniotic fluid is deleterious for the bowel and may lead to inflammation of the bowel wall resulting in the production of a yellow fibrous tissue named perivisceritis[21,22]. This perivisceritis is accompanied by edema, cellular infiltration of epithelial cells and the presence of macrophages in the bowel wall. Specific therapeutic strategies, including amnio-exchange as a prenatal treatment, may be developed to prevent the resulting more serious bowel damage. Amnio-exchange has been tried for many years in some centers[22]. Amnio-infusion during pregnancy consists in replacing the amniotic fluid with a saline solution in order to reduce the inflammation of the bowel due to its contact with the amniotic fluid. In animal studies, amnio-exchange reduces the inflammation of the bowel wall by eliminating inflammatory compounds. However, since no prospective and randomized studies with human fetuses have yet been realized, we do not use this technique and none of our babies benefited from amnio-infusion. Inflammation of the bowel at birth, on the basis of the aspect of the bowel wall, the presence of large amount of fibrin, the abnormal thickness of the bowel wall and the absence of bowel movement or contraction after stimulation, does not help to predict the outcome of GS, and does not seem to correlate with the degree of bowel recuperation or bowel damage. The condition of the externalized bowel loops can be difficult to evaluate, and its appreciation is largely subjective and without predictive value.

The recommended mode and timing of delivery remains a subject of debate[23-26]. Labor may be deleterious to the externalized bowel loops, and may entail the risk of membrane rupture and of infection. However, most authors found that caesarean delivery presented no significant benefit and did not improve the outcome of infants with GS. It is therefore reserved for obstetric indications or acute fetal emergencies often related to other organ failure. Preterm delivery, in order to limit the period of intrauterine damage of the bowel due to contact with the amniotic fluid, was of no benefit and did not lessen the morbidity of GS[25].

Surgery is performed in our hospital under emergency conditions, in order to close the abdominal wall as quickly as possible[9,27]. We think that early repair leads to a lower incidence of perivisceritis[28]. Coughlin JP *et al*[29] also observed an absence of inflammatory desquamation on the bowels of babies operated immediately after birth. Nevertheless, the surgical procedure may have to be delayed if further investigation for associated anomalies is required or if the child is too small for the operation. The surgical procedure may also have to be postponed if the intra-abdominal pressure during reintegration of the loops is too high (more than 20 mmHg) and would require high ventilation pressure, myorelaxant drugs and diuretics[30]. In these cases, abdominal closure is deferred, and the intestinal loops are protected with a silo during their progressive reintegration into the abdominal cavity[7]. The use of a suspended "silo" for a few days, allowing the gradual return of the viscera into the naturally growing abdominal cavity, makes it possible to close the abdominal wall without undue pressure and with a relatively low risk of intestinal damage.

All our patients required TPN for at least 10 d. In the course of prenatal counseling, parents should be made aware that their newborn will need TPN, and therefore the placement of a central venous line to provide adequate intake until oral nutrition is possible.

Our study also shows that a long period of hospitalization should be expected (mean hospitalization time of 54.7 d) and that, not surprisingly, a prolonged hospitalization is associated with a less favorable outcome. Parents should also be made aware that the length of the hospital stay will depend on how long the

bowel needs to rest and on the duration of total enteral feeding, two elements which depend primarily on clinical conditions.

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COMMENTS

Background

The authors performed a retrospective study covering the period from January 2000 to December 2007.

Research frontiers

The median duration of total parenteral nutrition (TPN) was clearly and significantly greater in the "atresia group", and the period of time before total milk feeding could be introduced was longer.

Innovations and breakthroughs

Associated with intestinal abnormalities are malrotation and a degree of short bowel: Volvulus, perforation and atresia may also be found.

Applications

This study also shows that a long period of hospitalization should be expected (mean hospitalization time of 54.7 d) and a prolonged hospitalization is associated with a less favorable outcome.

Terminology

The duration of TPN or the timing of introduction of normal feeding.

Peer-review

The authors showed that the predictive factor of babies with gastroschisis (GS) is intestinal atresia by analysing 60 babies with GS.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Role of anti-stromal polypharmacy in increasing survival after pancreaticoduodenectomy for pancreatic ductal adenocarcinoma

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Abstract

AIM: To investigate the survival impact of common pharmaceuticals, which target stromal interactions, following a pancreaticoduodenectomy for pancreatic ductal adenocarcinoma.

METHODS: Data was collected retrospectively for 164 patients who underwent a pancreaticoduodenectomy for pancreatic ductal adenocarcinoma (PDAC). Survival analysis was performed on patients receiving the following medications: angiotensin-converting enzyme inhibitors (ACEI)/angiotensin Ⅱ receptor blockers (ARB), calcium channel blockers (CCB), aspirin, and statins. Statistical analysis included Kaplan-meier survival estimates and cox multivariate regression; the latter of which allowed for any differences in a range of prognostic indicators between groups. Medications showing a significant survival benefit were investigated in combination with other medications to evaluate synergistic effects.

RESULTS: No survival benefit was observed with respect to ACEI/ARB (n = 41), aspirin or statins on individual drug analysis (n = 39). However, the entire CCB group (n = 26) showed a significant survival benefit on multivariate cox regression; hazard ratio (HR) of 0.475 (CI = 0.250-0.902, P = 0.023). Further analysis

revealed that this was influenced by a group of patients who were taking aspirin in combination with CCB; median survival was significantly higher in the CCB + aspirin group (n = 15) compared with the group taking neither drug (n = 98); 1414 d *vs* 601 d (P = 0.029, log-rank test). Multivariate cox regression revealed neither aspirin nor CCB had a statistically significant impact on survival when given alone, however in combination the survival benefit was significant; HR = 0.332 (CI = 0.126-0.870, P = 0.025). None of the other medications showed a survival benefit in any combination.

CONCLUSION: Aspirin + CCB in combination appears to increase survival in patients with PDAC, highlighting the potential clinical use of combination therapy to target stromal interactions in pancreatic cancer.

Key words: Pancreatic ductal adenocarcinoma; Stroma; Polypharmacy; Calcium channel blockers; Angiotensin Ⅱ receptor blockers; Angiotensin-converting enzyme inhibitors; Aspirin; Pancreaticoduodenectomy; Statins

Core tip: Stromal interactions play a large part in the dismal prognosis of pancreatic cancer. Recent laboratory studies have examined the potential use of common pharmaceuticals, such as calcium channel blocker (CCB), aspirin, angiotensin-converting enzyme inhibitors/angiotensin Ⅱ receptor blockers and statins, in inhibiting these protumorigenic stromal interactions. We retrospectively collected data from 164 patients whom underwent a pancreaticoduodenectomy to remove a pancreatic ductal adenocarcinoma, to see if the potential benefits of these drugs translated into increased survival. Our finding that those taking a combination of aspirin and CCB survived over twice as long as those on neither drug, highlights the potential of novel drug combinations to increase survival in pancreatic cancer.

Tingle SJ, Moir JA, White SA. Role of anti-stromal polypharmacy in increasing survival after pancreaticoduodenectomy for pancreatic ductal adenocarcinoma. *World J Gastrointest Pathophysiol* 2015; 6(4): 235-242 Available from: URL: http://www.wjgnet.com/2150-5330/full/v6/i4/235.htm DOI: http://dx.doi.org/10.4291/wjgp.v6.i4.235

INTRODUCTION

Pancreatic cancer is one of the most aggressive malignancies with a dismal prognosis. In the United Kingdom it is the 5th most common cause of cancer death, with 1- and 5-year survival rates of 20.8% and 3.3% respectively[1]. The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), making up 95% of cases. Given this poor prognosis, with little improvement over the last 40 years, novel options for therapeutic targets are being investigated, in both the palliative setting to improve survival and the post-resection setting to reduce recurrence rates. One such target is the complex interaction between pancreatic cancer and the surrounding tissue, which is termed stroma.

The stroma is the local microenvironment which surrounds the tumour and is made up of a variety of cellular (vascular, inflammatory and neural cells) and non-cellular components. Most of these are present in the normal pancreas and aid in regulating normal pancreatic function. In the presence of a pancreatic tumour stromal cells become activated, resulting in a desmoplastic reaction that increases tumour proliferation, chemotherapy resistance and metastasis[2-5]. PDAC has the most significant interactions with surrounding stroma out of all solid organ epithelial cancers, which may partly explain the aggressive nature of the disease, and as such is currently a hot topic in pancreatic cancer research.

Patients who receive surgery benefit from improved outcomes, but surgical resection is only an option in around 20% of patients[6]. Previous studies have shown that despite the curative intent of surgery, the majority of patients experience recurrence[7]. This is largely due to incomplete R1 resection. However, the activated stroma which is left behind in the remnant pancreas, even in theoretically complete R0 resections, may have a role in creating a protumorigenic environment and encouraging recurrence of disease.

Various scientific studies have demonstrated that commonly used pharmaceutical agents may influence the protumorigenic cancer-stroma relationship. Calcium channel blockers (CCB)[8], aspirin[9], statins[10,11], angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB)[12,13] demonstrate inhibitory effects on stromal interactions, manifesting as reduced growth and/or metastasis of PDAC cells in a mixture of *in-vitro* and animal studies. This effect is enhanced in combination with gemcitabine (the current first line chemotherapeutic agent in pancreatic cancer), suggesting that these medications may work by improving chemo penetrance[9,14].

ACEI and ARBs, which affect stromal interactions *via* the local renin-angiotensin system (RAS), have been shown to improve survival[15]. Furthermore aspirin[16] and statins[17] have been shown to reduce the risk of pancreatic cancer development, suggesting an inhibitory effect on carcinogenesis. The anticancer potential of these drugs has been examined in a whole range of other cancer types[18-21].

This study aims to investigate whether the aforementioned laboratory findings translate into a significant clinical survival benefit in the post-resection setting, and to observe if any of these medications could act in combination to give a synergistically beneficial effect on survival.

Table 1 Differences in Kaplan-Meier estimated median survival between individual drugs groups

Drug name	Number taking the drug out of 164 patients	Median survival estimate for those taking the drug (d)	Median survival for those not taking the drug (d)	*P* value (log rank test)
ACEI/ARB	41	539	611	0.652
CCB	26	815	528	0.061
Aspirin	55	504	546	0.846
Statins	39	504	577	0.368

Significance calculated using log-rank tests. ACEI/ARB: Angiotensin-converting enzyme inhibitors/angiotensin Ⅱ receptor blockers; CCB: Calcium channel blockers.

MATERIALS AND METHODS

Patients

All patients included in the study had a histologically confirmed PDAC removed from the head of the pancreas by Whipple's pancreaticoduodenectomy between December 2004 and March 2013. Data was retrospectively collected from hand held and electronic patient notes. This included whether they were taking ACEI/ARB (which were grouped as they both affect the local RAS), CCB, aspirin or statins as regular medications upon discharge after their operation.

Any drug which offered a significant benefit in survival was then investigated in combination with the other drugs to determine if any synergistic benefits were present.

Statistical analysis

Kaplan-Meier was used to calculate estimated median overall survival, which was measured in days after surgery, and the log-rank test was applied to compare groups. As some of the patients were still alive at the end of the study, censoring was applied, allowing these patients to be included in the analysis. χ^2 test was used to compare categorical variables. A $P < 0.05$ was considered significant.

Cox regression was used to exclude possible cofounding factors, and estimate the hazard ratios for various drug groups, adjusting for prognostic indicators. Prognostic indicators included sex, age (< 60 or ⩾ 60 years), blood pressure status (hypertensive or normotensive), pre-operative body mass index (< 18.5, 18.5-25, > 25), post-operative adjuvant chemotherapy, CA19-9 level at diagnosis (< 47, 47-1000, > 1000), American Society of Anesthesiologists (ASA) grade (1-2 or 3-4), resection margin status and TNM staging.

SPSS was used for all of the statistical analysis.

RESULTS

In total, 195 patients had a Whipple's pancreaticoduodenectomy to remove a PDAC at the Newcastle Freeman Hospital between December 2004 and March 2013. Of these data could be collected for 164 patients with a median follow up time of 23.9 mo.

Individual drug analysis

Drugs were initially looked at on an individual basis, creating four groups; ACEI/ARB (n = 30/11 = 41), CCB (n = 26), aspirin (n = 55), and statins (n = 39). Median daily dose of the various drugs were as follows; aspirin 75 mg, CCB 10 mg (range: 5-180 mg), statin 40 mg (5-40 mg), ACEI 10 mg (1.25-40 mg) and ARBs 60 mg (4-300 mg). Information on adjuvant chemotherapy could be collected for 153 patients. In total 110 (71.9%) received post-operative adjuvant chemotherapy. Of these 53 (48.2%) received 5FU treatment in the MAYO regime, 53 received Gemcitabine (48.2%), and the remaining 4 (3.6%) received other chemotherapeutic agents. Of the 53 patients taking Gemcitibine, 4 were also receiving Capecitabine and 2 were also receiving Carboplatin. None of the patients received radiotherapy.

Initial analysis compared median survival of every patient taking a particular drug, with those not taking that drug (Table 1). This initial analysis did not investigate whether the drug was being taken in combination with any of the other medications. None of the medications showed a statistically significant impact on survival when a Log rank test was applied. The only drug which showed an increase in median survival was CCB, (Figure 1) with those taking the drug having a median survival of 815 d compared with 528 d in those not taking the drug (P = 0.061). At this stage, the CCB group included every person taking CCB, some of which were also taking other medications such as aspirin, statins or ACEI/ARBs in various combinations.

When multivariate analysis was applied, being in the CCB group was an independent predictor of improved survival with a hazard ratio of 0.475 (P = 0.023) as can be seen in Table 2. All of the other drugs resulted in worsened survival, but this was not statistically significant.

Combination therapy

After observing a statistically significant benefit in the entire CCB group, this drug was analysed in combination with the other drugs in the study, as seen at the top of Table 3. Both Kaplain-Meier median survival estimates and multivariate cox regression showed that there was no significant survival benefit in people taking either statins or ACEI/ARBs along with CCB (Table 3). However, the CCB + aspirin group (n = 15) had a significantly improved median survival; 1414 d compared to 528 d in those not on this drug combination (P = 0.012 Log rank test). This benefit was confirmed in the multivariate cox regression analysis; being in the CCB + aspirin group

Table 2 Univariate and multivariate cox regression comparing individual drug groups with those not taking the drug

Drug name	Number taking the drug out of 164 patients	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
ACEI/ARB	41	1.094 (0.741-1.614)	0.653	1.129 (0.617-2.065)	0.693
CCB	26	0.635 (0.393-1.025)	0.063	0.475 (0.250-0.902)	0.023
Aspirin	55	1.036 (0.726-1.479)	0.846	1.041 (0.651-1.667)	0.865
Statins	39	1.200 (0.806-1.787)	0.369	1.055 (0.614-1.814)	0.845

ACEI/ARB: Angiotensin-converting enzyme inhibitors/angiotensin Ⅱ receptor blockers; CCB: Calcium channel blockers.

Table 3 Kaplan-Meier survival estimates and multivariate cox regression comparing patients taking a combination of medications with patients on one or neither drug

Drug combination	Number of people on combination out of 164	Kaplan-Meier estimated median survival			Multivariate cox regression	
		For those on the drug combination	For those not on drug combination	*P*-value (log rank)	HR (95%CI)	*P*-value
CCB + aspirin	15	1414	528	0.012	0.300 (0.122-0.735)	0.008
CCB + statin	12	544	539	0.284	0.413 (0.155-1.101)	0.077
CCB + ACEI/ARB	12	485	541	0.450	0.512 (0.194-1.348)	0.175
Aspirin + statin	27	504	546	0.697	0.969 (0.509-1.844)	0.924
Aspirin + ACEI/ARB	22	485	569	0.923	0.948 (0.438-2.054)	0.893
Statin + ACEI/ARB	21	368	577	0.426	1.126 (0.533-2.379)	0.756

ACEI/ARB: Angiotensin-converting enzyme inhibitors/angiotensin Ⅱ receptor blockers; CCB: Calcium channel blockers.

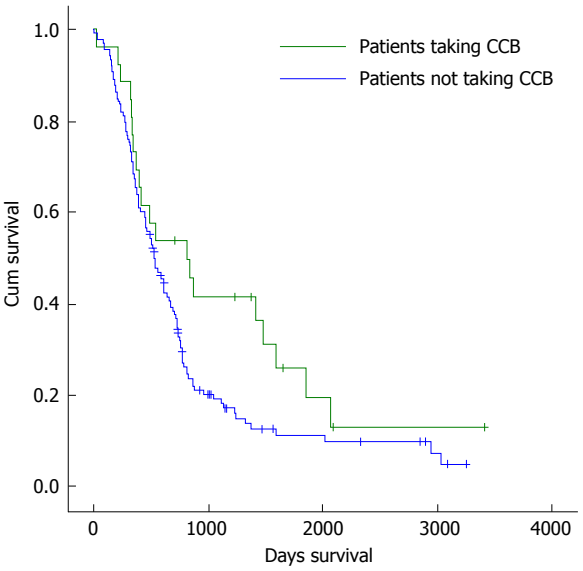

Figure 1 Kaplan-Meier curves showing overall survival in those taking calcium channel blockers and those not taking the drug. (*P* = 0.061 using log rank test). CCB: Calcium channel blockers.

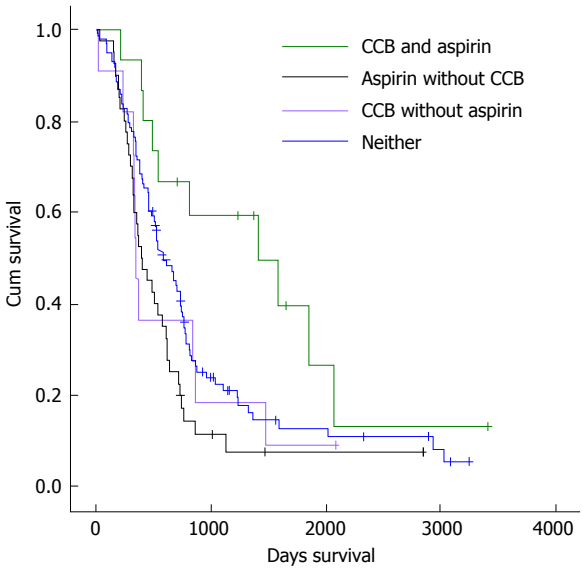

Figure 2 Kaplan-Meier curves showing overall survival in those taking calcium channel blockers and aspirin in combination and taking one or neither drug. (*P* = 0.01 using log rank test). CCB: Calcium channel blockers.

gave a HR of 0.300 (CI = 0.122-0.735, *P* = 0.008). Further analysis later revealed that this CCB + aspirin group was solely responsible for the increase in median survival seen in the initial entire CCB group (Figure 2). No other combination of ACEI/ARB, statins or aspirin showed a significant improvement in survival as seen in Table 3.

CCB and aspirin

Further statistical analysis of patients taking CCB and/or aspirin was then performed. This divided the 164 patients into four groups; those taking CCB + aspirin in combination (*n* = 15), those taking aspirin without CCB (*n* = 40), those taking CCB without aspirin (*n* = 11), and those taking neither drug which acted as the control group (*n* = 98). χ^2 tests were then used to compare differences in the various prognostic indicators between these drug groups (Table 4). None of these prognostic indicators showed statistically significant differences between groups, except blood pressure status, ASA grade, and resection value; those taking CCB and/or aspirin were more likely to suffer from hypertension (*P*

Table 4 Comparison of differences in prognostic indicators between drug groups

Characteristics	No CCB/aspirin, *n* (%) *n* = 98	CCB and aspirin, *n* (%) *n* = 15	Aspirin without CCB, *n* (%) *n* = 40	CCB without Aspirin, *n* (%) *n* = 11	*P* value
Sex					
Male	56 (57.1)	5 (33.3)	25 (62.5)	8 (72.7)	0.169
Age (yr)					
< 60	39 (39.8)	5 (33.3)	8 (20.0)	3 (27.3)	0.157
⩾ 60	59 (60.2)	10 (66.7)	32 (80.0)	8 (72.7)	
Blood pressure status					
Hypertensive	27 (27.6)	13 (86.7)	22 (55.0)	11 (100.0)	0.000
Non-hypertensive	71 (72.4)	2 (13.3)	18 (45.0)	0 (0.0)	
BMI					
< 18.5	2 (2.0)	0 (0)	1 (2.5)	0 (0.0)	0.307
18.5-25	52 (53.1)	5 (33.3)	24 (60.0)	3 (27.3)	
> 25	41 (41.8)	9 (60.0)	14 (35.0)	8 (72.7)	
Adjuvant chemotherapy					
Received post-op	69 (70.4)	9 (60.0)	23 (57.5)	9 (81.8)	0.333
Not received	24 (24.5)	5 (33.3)	13 (32.5)	1 (9.1)	
CA19-9					
< 47	27 (27.6)	3 (20.0)	9 (22.5)	1 (9.1)	0.437
47-1000	51 (52.0)	7 (46.7)	20 (50.0)	7 (63.6)	
> 1000	8 (8.2)	3 (20.0)	6 (15.0)	3 (27.3)	
ASA grade					
1-2	81 (82.7)	12 (80.0)	22 (55.0)	8 (72.7)	0.008
3-4	17 (17.3)	3 (20.0)	18 (45.0)	3 (27.3)	
Resection value					
R0	14 (14.3)	6 (40.0)	9 (22.5)	5 (45.5)	0.020
R1	83 (84.7)	9 (60.0)	31 (77.5)	6 (54.5)	
T status					
T1-2	3 (3.1)	2 (13.3)	1 (2.5)	0 (0.0)	0.199
T3-4	95 (96.9)	13 (86.7)	39 (97.5)	11 (100.0)	
N status					
N0	7 (7.1)	3 (20.0)	5 (12.5)	0 (0.0)	0.242
N1	90 (91.8)	12 (80)	35 (87.5)	11 (100.0)	

P-values were calculated using tests χ^2 tests. CCB: Calcium channel blockers; ASA: American society of anaesthesiologists; BMI: Body mass index.

Table 5 Kaplan-Meier survival estimates and multivariate cox regression comparing patients taking a combination of calcium channel blockers + aspirin with patients on one or neither drug

Drug group	Number of people in group	Estimated median survival (d)	*P*-value (log rank) compared to control	Multivariate cox regression HR (95%CI)	*P*-value
Control (no CCB/aspirin)	98	601	-	1	-
CCB + aspirin	15	1414	0.029	0.332 (0.126-0.870)	0.025
Aspirin without CCB	40	392	0.032	1.658 (0.968-2.840)	0.066
CCB without Aspirin	11	343	0.563	1.039 (0.416-2.595)	0.935

CCB: Calcium channel blockers.

= 0.000), more likely to have a higher ASA grade (*P* = 0.008), and more likely to have a successful surgical resection (*P* = 0.020).

Kaplan-Meier estimated median survival was 601 d in those taking neither drug (Table 5). At 1414 d, combination of CCB + aspirin made a statistically significant improvement in median survival (*P* = 0.029 log rank test). Taking either drug alone led to a decrease in median survival time; median survival in the aspirin without CCB group was 392 d (*P* = 0.032), and was 343 d in the CCB without aspirin group (*P* = 0.563). Differences in survival between groups can be seen in Figure 2.

The previously observed benefit of taking CCB + aspirin remained statistically significant when multivariate cox regression was used; this allowed for any differences in prognostic indicators, including resection status, and compared the CCB + aspirin group with those taking neither drug, to find a hazard ratio of 0.332 (CI = 0.126-0.870, *P* = 0.025). Taking either of the drugs in isolation made no statistically significant impact on survival when multivariate cox regression was applied.

DISCUSSION

This study interestingly demonstrates a greater than twofold increase in post-operative median survival in patients who take a combination of CCB and aspirin, as compared to those taking neither drug. The estimated

median survival in patients taking neither drug was comparable to that in similar studies[22-24]. These observations remained significant when allowing for a range of prognostic indicators using multivariate cox regression. In contrast taking any of these medications in isolation or other combinations did not impact on survival. One may therefore postulate that aspirin and CCB's may act in synergy to inhibit cancer-stromal interactions and thus improve survival.

It has been suggested that the dense desmoplastic reaction that surrounds tumours may account for up to 90% of tumour volume[25]. This represents an intriguing concept in tumour staging, as whilst one may theoretically achieve a tumour-free R0 resection margin, large amounts of activated tumour stroma may be left behind and act as a catalyst for recurrent disease. Therefore, in the context of this study's findings, it may be that aspirin and CCB act in combination to inhibit any subsequent protumorigenic activity, thus reducing/slowing recurrence and improving survival.

A vast array of different signalling pathways exist which are involved in the development and progression of cancer. The benefits of inhibiting multiple pathways, or multiple points on a single pathway, *via* combination drug therapy is supported by clinical data showing the synergistic effects of combining anti-cancer therapies leading to improved outcomes compared to the sum of each individual drug's benefits[26]. To the author's knowledge, this is the first study looking at a combination of CCB and aspirin as a therapeutic option in pancreatic cancer. As a result, the mechanisms of action are poorly understood. However, we can consider some of the laboratory work which prompted this study, to appreciate some of the potential underlying mechanisms.

Aspirin's role as an anti-inflammatory, anti-platelet drug is well established through its inhibitory action on the inflammatory enzyme cyclooxygenase-1 (COX-1), and is known to have a key role in reducing the risk of cancer development in a variety of malignancies, including pancreatic cancer[16,27,28]. The mechanism of effect is likely due to the inhibition of stromal-interactions which interfere with local inflammation. This is particularly pertinent in PDAC given the significant inflammatory environment observed, with a weak and fragile extracellular matrix promoting cancer development[29]. The fact chronic pancreatitis is a key risk factor in PDAC supports this.

There are various pathways aspirin exerts an influence upon in this setting. Incorrect regulation of the transcription factor nuclear factor kappa B (NF-κB) can lead to excess local inflammation and a positive feedback loop amplifying the activity of the local RAS to oncogenic levels[30]. NF-κB is frequently activated in pancreatic cancer which suggests a link between local inflammation and progression of pancreatic cancer[31]. Aspirin's inhibitory effect on inflammation and NF-κB have been demonstrated in laboratory studies[31,32], and a resulting decrease in the progression and development of PDAC has been observed in mouse models[33].

Another molecule involved in inflammation is COX-2, an inflammatory enzyme which is also often raised in pancreatic cancer; the inhibition of which leads to decreased carcinogenesis[34]. Although aspirin has a greater effect on COX-1, it may have a role in inhibiting COX-2 in pancreatic cancer. The immune system also plays a role in inflammation, and immune inflammatory cells are one of the cellular components of pancreatic stroma. One such immune cell is the FOXP3 regulatory T cell, which aspirin has been shown to inhibit in the context of pancreatic stroma[9].

CCBs have also shown promise in the laboratory, with an earlier study showing that CCBs can inhibit growth and decrease the doubling time of pancreatic cancer cells[35]. Furthermore the stroma is known to represent a barrier to chemotherapy, and CCBs may have a role in improving chemo penetrance in a range of cancer types, including pancreatic[36]. CCBs have been shown to increase the effectiveness of chemotherapy on a resistant pancreatic adenocarcinoma *via* its effect on P-glycoprotein, which is also known as multidrug resistance protein[37].

Another possible mechanism of action involves cholecystokinin (CCK), an intracellular peptide hormone which has various roles in control of the pancreas[38]. It is known that high levels of CCK can cause both formation and progression of pancreatic cancer[39]. CCBs have the ability to limit the effects of CCK on pancreatic cells and lead to decreased carcinogenesis[40] and metastasis[41]. Alternatively CCBs have been shown to inhibit the proliferation of pancreatic cancer through the blockade of IK calcium-activated potassium channels[8].

This study is limited by the small sample size of patients taking aspirin and CCB in combination. It is also limited by the fact we looked at regular medications being taken on discharge from hospital, which did not allow any analysis into the effect of altering the duration of administration of these medications.

The retrospective nature of this work brings an inherent selection bias however this was countered through multivariate analysis including a range of prognostic indicators. The one key difference between groups related to the resection margin status, where those taking aspirin and CCB in combination were more likely to have an R0 resection. However, when allowed for using multivariate analysis, the benefits of combining aspirin and CCB still remained statistically significant. One may potentially hypothesise that the anti-stromal effects of taking this combination of medications pre-operatively led to a less locally advanced tumour and therefore a higher chance of full resection.

It could be argued that it is simply the CCB which are having an effect on survival, as seen in our initial individual drug analysis, and that aspirin was only found as a coincidence as we were looking at combinations in the already beneficial CCB group. However, our statistics would suggest that the only reason that CCB showed a benefit on individual drug analysis was the presence of 15 people within the group who were also

taking aspirin. Indeed, without those 15 patients, CCB in isolation showed no benefit.

In conclusion, this novel retrospective study has shown that the potential anti-stromal benefits of CCB and aspirin demonstrated by previous laboratory studies do translate into survival benefits in patients with pancreatic ductal adenocarcinoma. Laboratory studies would be useful to determine the mechanism of action of the synergistic effect observed. Further clinical studies with larger patient groups, as well as randomised prospective studies, will help to determine the true anti-cancer potential of these drugs. This study builds on previous laboratory research and represents an exciting new range of potential therapeutics for pancreatic cancer, especially given the cheap, accessible and safe nature of these drugs.

COMMENTS

Background

The complex interaction between pancreatic ductal adenocarcinoma and its surrounding tissue microenvironment (termed stroma) plays a large part in the dismal prognosis of pancreatic cancer. Recent laboratory studies have examined the potential use of common pharmaceuticals, such as calcium channel blockers (CCB), aspirin, angiotensin-converting enzyme inhibitors/angiotensin Ⅱ receptor blockers and statins, in inhibiting these protumorigenic stromal interactions. Further clinical research is required to look at the effects of these drugs on mortality.

Research frontiers

Studies looking at whether the potential benefits suggested by laboratory research translates into increased survival in clinical research is a current hotspot in this field. There has also been growing interest into the effect of combining therapies to get a synergistic effect; an area which this study explores.

Innovations and breakthroughs

This study built on previous laboratory research to show survival benefits in the clinical setting. The authors demonstrated a statistically significant improvement in Kaplan-Meier estimated median survival in patients taking a combination of aspirin and CCB, a combination which has not been studied in this setting before. The twofold increase in estimated median survival seen in the aspirin + CCB group was confirmed by multivariate cox regression which found the increase in survival to remain significant when a range of prognostic indicators was allowed for.

Applications

If the findings of this study are confirmed by further research, patients with pancreatic cancer could expect improvements in life expectancy, with the simple addition of extremely cheap, well tolerated, and readily available medications.

Terminology

The stroma is the local microenvironment which surrounds the tumour and is made up of a variety of cellular (vascular, inflammatory and neural cells) and non-cellular components. Most of these are present in the normal pancreas and aid in regulating normal pancreatic function. In the presence of a pancreatic tumour stromal cells become activated, resulting in increased tumour proliferation, chemotherapy resistance and metastasis.

Peer-review

This is a novel look at a very interesting topic. In the clinical finding presented in this manuscript, the authors showed that combination CCB and aspirin can increase survival in patients with pancreatic cancer pancreatic ductal adenocarcinoma following pancreaticoduodenectomy although in a small number of patients. A potential mechanism related to targeting stromal interactions in pancreatic cancer was proposed.

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CASE REPORT

Energetic etiologies of acute pancreatitis: A report of five cases

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Abstract

There are several common causes of acute pancreatitis, principally excessive alcohol intake and gallstones, and there are many rare causes. However, cases of pancreatitis still occur in the absence of any recognizable factors, and these cases of idiopathic pancreatitis suggest the presence of unrecognized etiologies. Five cases of acute pancreatitis in four patients came to attention due to a strong temporal association with exposure to nerve stimulators and energy drinks. Given that these cases of pancreatitis were otherwise unexplained, and given that these exposures were not clearly known to be associated with pancreatitis, we performed a search for precedent cases and for mechanistic bases. No clear precedent cases were found in PubMed and only scant, weak precedent cases were found in public-health databases. However, there was a coherent body of intriguing literature in support of a mechanistic basis for these exposures playing a role in the etiology of pancreatitis.

Key words: Pancreatitis; Energy drinks; Transcutaneous electric nerve stimulation; Etiology; Chronic pain

Core tip: This may be the first report of nerve stimulators or energy drinks playing an etiologic role in the development of pancreatitis. Five recent cases of otherwise unexplained pancreatitis recently came to attention due to a strong temporal association between pancreatitis and exposure to nerve stimulators (3 cases in 3 patients) and energy drinks (2 cases in 1 patient).

Although causality is not shown, the temporal association is striking.

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INTRODUCTION

Acute pancreatitis is a common and potentially life-threatening disease, whose incidence is increasing[1]. While the etiology of the vast majority of cases is identified as either alcohol or biliary stones, many cases (10%-34%)[1,2] are labeled idiopathic or cryptogenic, due to unknown etiology, and this proportion too is increasing[2,3]. Other, less common causes of pancreatitis that are identifiable include hypertriglyceridemia, tumors and stones, autoimmune diseases, and medications.

Use of energy drinks and nerve stimulators is also on the rise. Use of energy drinks such as RedBull and RockStar is increasing due in part to aggressive marketing campaigns and other psychosocial factors. The range and relative concentrations of their various ingredients vary widely, and per capita consumption has recently doubled in the United States[4-6]. Electrical nerve stimulation, such as transcutaneous electrical nerve stimulation (TENS) are employed at various intensities and frequencies for pain suppression, a controversial use[7].

Hypothesis

Very few data on idiopathic or cryptogenic causes of pancreatitis are available. Some cases of so-called idiopathic pancreatitis are likely due to unrecognized, or poorly understood, genetic defects[3,8-13] and pancreaticobiliary malformations[14,15]. Yet, there still remains a group of patients with so-called true idiopathic pancreatitis (TIP), whose etiology cannot be identified[16]. Three such patients with seemingly TIP, with no identified cause, were found to have a clear temporal association with the use of nerve-stimulation devices, and a fourth such patient was found to have a clear temporal association between 2 separate episodes of pancreatitis and consumption of the popular energy drink, Rockstar™ (Table 1). Other causes of pancreatitis, including gallstones, alcohol, autoimmune pancreatitis (elevated IgG4 levels), and hypertriglyceridemia, were ruled out, leading to the hypothesis that exposures to nerve stimulation or to energy drinks could play a role in the etiology of acute pancreatitis.

CASE REPORT

Three nerve-stimulation cases

A healthy female in her 40s underwent cholecystectomy for suspected biliary pancreatitis based on a suspicion of gallbladder sludge, but then re-presented several months later with recurrent pancreatitis. Upon further questioning it became clear that she occasionally used TENS to treat pain, including prior to her current episode of interstitial edematous pancreatitis. She did not recall if she used it prior to her pre-cholecystectomy pancreatitis. She responded to standard nonoperative therapy and recovered well.

A healthy female in her 30s, with an implanted electrical nerve stimulator device for chronic back pain, developed interstitial edematous pancreatitis. She responded to standard nonoperative therapy and recovered well.

A healthy female in her 50s wore a TENS device for back pain during a 10-h car trip. Shortly after arrival she developed severe, extensive, necrotizing pancreatitis and disconnected-duct syndrome, requiring necrosectomy, after which she recovered well. Although following her stay in the intensive-care unit, and her extensive operation and requisite recovery, she could not recall the exact settings she used for TENS, but thought that it was a moderate setting and that the pads were applied to her back for the vast majority of her 10-h car trip. Pathology following operation revealed extensive necrotic pancreatic and peripancreatic tissue.

Two energy-drink cases

A healthy male in his 40s with a remote history of severe acute alcoholic pancreatitis with pseudocyst formation, all now resolved. He was thought certainly to be free of recent alcohol use, but developed 2 episodes of interstitial edematous pancreatitis, both following Rockstar™ consumption. He responded well to nonoperative therapy.

Literature search

To inquire whether these could be two heretofore unappreciated etiologies of acute pancreatitis, a literature search was performed in PubMed looking for a mechanistic basis, using the title and abstract terms "pancreatitis" with either "idiopathic" or "etiology" and combined this with either "energy drink" or "nerve stimulation". In addition, a search was performed of www.fda.gov for pancreatitis, nerve stimulation, energy drink, and several ingredients in common energy beverages (*e.g.*, milk thistle, guarana, ginkgo, ginseng)[17].

The PubMed search revealed no case reports associating pancreatitis with either energy drinks or with nerve stimulation. Although energy drinks are associated with a variety of adverse signs and symptoms, such as nausea, vomiting, diarrhea, abdominal pain, hyperhidrosis, tachycardia, irritability, insomnia, stroke, and psychotic and bipolar disorders, the Food and Drug Administration (FDA) search on www.fda.gov revealed only one report of pancreatitis (which required hospitalization) associated with the energy drink Redbull™[18] and two reports of pancreatic disorders associated with 5-Hour Energy Booster™ and Monster Energy™[19]. Four

Table 1 Patient characteristics

Pt	Age	Gender	Type of exposure	Time gap between exposure and pancreatitis	Duration of exposure	Severity of pancreatits	TG	IgG4	Confounding factors
1	40s	F	TENS	Narrow	Short	Mild	Nl	Nl[3]	CCB and PPI
2	30s	F	IENS	Narrow	Unk	Mild	Unk	Unk	Unk[2]
3	50s	F	TENS	Narrow	Long	Severe	Nl	Nl[3]	None[1]
4	40s	M	Energy drink	Narrow	Consumed Rockstar™ prior to 2 unexplained episodes	Both mild	Nl	Nl	Initial episode due to alcohol, but abstinent for 1 yr prior to these 2 episodes

[1]No medications, no alcohol, no gallstones (by transabdominal and intraoperative, but not endoscopic, ultrasound), there was no evidence for or against genetic causes and pancreas divisum; [2]No alcohol, and no gallstones (by transabdominal but not endoscopic ultrasound), but the patient could not recall her medication history; [3]Total IgG, not IgG4, was sent. Pt: Patient; Nl: Normal; Unk: Unknown; TG: Triglyceride level; TENS: Transcutaneous electrical nerve stimulation; F: Female; M: Male; CCB: Calcium-channel blocker; PPI: Proton-pump inhibitor; IENS: Implnted electrical nerve stimulator.

Table 2 List of observations associating electrical nerve stimulations and the development of acute pancreatitis

	Ref.
Observation	
All cases reported here were considered idiopathic	N/A
There was a strong temporal association of exposure and AP	N/A
There was a directly proportional relationship between duration of exposure and severity of subsequent AP	N/A
Neurogenic inflammation is increasingly recognized to play a role in development of AP, with sensory nerves in particular being considered a final common pathway in AP	[24,30,39,40]
TRPV1 and TRPA1 expression and function in pancreatic afferent neurons increases and blocking this pathway attenuates pancreatitis in a mouse model of AP	[25,26]
Over-stimulation of nerves associated with pancreatic disease (decrease in pancreatic blood flow and DNA synthesis) in rats	[34]
Neural cross talk between the duodenum and pancreas (duodeno-pancreatic reflex at T6-T13) can promote AP in a rat model	[41]
Possibly contradictory observations	
Stimulation by electroacupuncture of dorsal segmental points corresponding to levels that innervate pancreas (by splanchnic nerves; T9-T11) causes decrease in fasting blood glucose	[33]
Electroacupuncture protects against CCK-induced AP in rats	[31]
Electroacupuncture to paraumbilical point ST25 (dermatome T10) down-regulates pro-inflammatory cytokines (TNFα, IL-6) and attenuates the morphological damage to pancreas in a rat model of AP	[32]

AP: Acute pancreatitis; N/A: Not available; TNF: Tumor necrosis factor; IL- 6: Interleukin- 6; TRPV1: Transient receptor potential vanilloid 1; TRPA1: Transient receptor potential cation channel, subfamily A, member 1; CCK: Cholecystokinin.

reports of pancreatitis associated with ginkgo were found in www.fda-reports.com.

DISCUSSION

The observation of five cases of pancreatitis in 4 patients without the usual risk factors for pancreatitis, but with other exposures, which themselves were notable, either because the exposure was unusually prolonged (patient #3, with exposure to nerve stimulation during the entirety of a 10 h car ride), or unusually recurrent (patient #4, with pancreatitis episodes occurring following each consumption of the energy drink Rockstar™) led us to hypothesize that these could conceivably be unrecognized etiologies of pancreatitis. Although some of the patients in Table 1 has confounding factors, such as medications loosely associated with AP, and pancreas divisum and genetic causes were not possible to rule out in each and every case, nevertheless it is striking that the patient with the most severe pancreatitis had the purest form of seemingly TIP (lacking any identifiable confounders) had the greatest exposure to TENS, and had the most severe pancreatitis, necrotizing pancreatitis requiring multidisciplinary management[20]. Our search of the literature and FDA records revealed no published case reports and only scant FDA evidence, but did reveal supporting evidence of this hypothesis worthy of discussion.

Nerve stimulation and neurogenic inflammation

Ironically, nerve stimulation, such as TENS, has been used to treat pain of pancreatitis, as well as pain of many other sources[21-23] but the number of patients is too small, and the follow-up too inconsistent, for there to be any observed causal relationship between pancreatitis and nerve stimulation. Certainly it is well known that the pancreas is richly innervated, and mounting evidence suggests that pathologic activation of pancreatic neurons and the inflammatory sequelae of that activation (known as neurogenic inflammation) play a role in the development of pancreatitis (Table 2). The concept of neurogenic inflammation is additionally supported by the observation that most of the neurotransmitters of C and Aδ fibers of the pancreas have proinflammatory

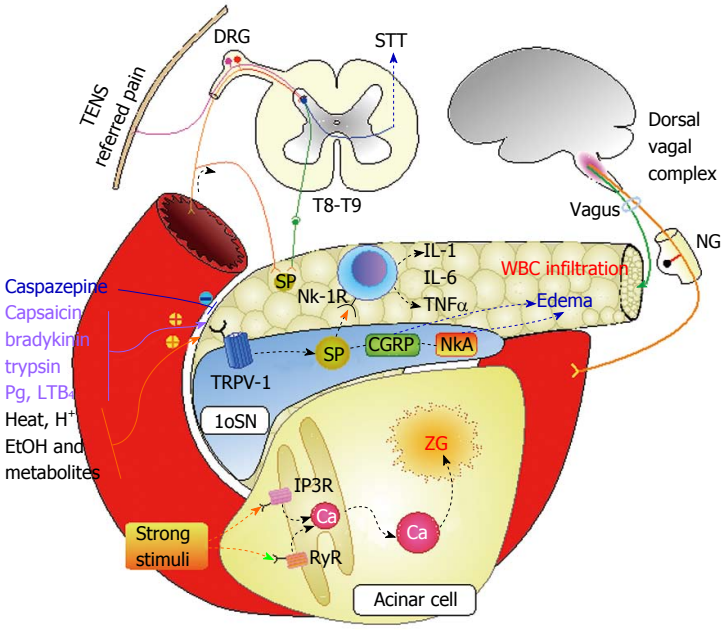

Figure 1 Schematic model illustrating possible mechanisms of neurogenic pancreatitis. AP: Acute pancreatitis; STT: Spinothalamic tract; TENS: Transcutaneous electrical nerve stimulation; DRG: Dorsal root ganglion; IL: Interleukin; TNF: Tumor necrosis factor; NK-1R: Neurokinin receptor 1; 1oSN: Primary sensory neuron; NG: Nodose ganglion; Pg: Prostaglandins; SP: Substance P; TRPV-1: Transient receptor potential vanilloid 1; CGRP: Calcitonin gene related protein; NkA: Neurokinin A; IP3R: Inositol-3-phosphate receptor; RyR: Ryanodine receptor.

actions[24].

Further intriguing experimental evidence exists linking neurogenic inflammation and pancreatitis. For example, sensory neurons of the pancreas express channels whose activation induces pancreatic inflammation, and whose blockade attenuates experimental pancreatitis[25-27]. Similarly, in a study of the modulatory role of bradykinin in neurogenic inflammation, a potent inhibitor of bradykinin was administered in an animal model of pancreatitis and this administration attenuated the hypotension, edema, and hypovolemia associated with the pancreatitis, suggesting modulation of nerve stimulation modulates severity of pancreatitis[28].

Studying the neuropeptide substance P (SP), a common neurotransmitter mediating pain and other nerve signals, Figini *et al*[29] found that administration of SP to mice stimulated plasma extravasation from postcapillary venules in the pancreas, and that this effect was blocked by the administration of antagonists to the SP receptor. More recently, in a neonatal model of pancreatitis, administration of the neuron-denervating agent capsaicin significantly reduced histological severity scores and abolished plasma extravasation associated with pancreatitis[30], findings which support the notion that primary sensory neurons constitute a common final pathway for pancreatitis.

Figure 1 illustrates in a schematic model some of these possible processes contributing to a neurogenic etiology of pancreatitis. Various stimuli may excite vanilloid receptor TRPV1 on primary sensory neurons (SN), which play the pivotal role in initiation of neurogenic inflammation. Subsequent depolarization release various neuropeptides, such as SP, neurokinin A, and calcitonin gene related peptide. The result is edema of pancreatic tissue due to massive vasodilation and white blood cells infiltration. Remarkably, those changes in pancreas may be caused by irritation of SN in duodenal mucosa, because of axonal dichotomy and central convergence at spinal or higher levels (Table 2). Damage to pancreatic acinar cells may also be mediated by non-specific excitation of ryanodine or inositol-3-phosphate receptors with subsequent calcium release from endoplasmic reticulum and activation of enzymes in zymogen granules inside the cell.

Interestingly, there is also evidence that some forms of nerve stimulation, such as electroacupunture, seem to be protective against pancreatitis (Table 2)[31-33]. However, the concept of dose may well explain why a small amount of stimulation can have the opposite effect as a large amount, as is commonly observed in medicine, when a small amount of an exposure is safe and effective, such as acetaminophen (paracetamol) in therapeutic doses working as a safe antipyretic and analgesic, but a higher dose is toxic and can cause liver failure and death. Perhaps a very high dose of TENS, as in patient #3, can cause severe acute necrotizing pancreatitis, known to require prolonged multidisciplinary management[20], where as a small dose may be protective, and a moderate dose may cause only a minor episode of pancreatitis.

Indeed, experimental neurochemical precedent exists for the notion that over-stimulation of nerves may cause pancreatic disease. In conscious rats, for example, stimulation of sensory nerves with low-dose capsaicin reduces basal pancreatic secretory function, while moderate doses increase this function, and large (neurotoxic) doses cause a 27% decrease in pancreatic blood flow accompanied by a decrease in DNA synthesis

in pancreatic tissue[34].

Energy drinks

Several aspects of the increasingly popular energy drinks are concerning. For example, because many energy drinks contain "natural" ingredients, such as ginseng, ginkgo, milk thistle, guarna-seed extract[17], these drinks are regulated as dietary supplements and not as medications, freeing manufacturers from the usual transparency associated with FDA-regulated products[6]. Similarly, several of the ingredients have been linked to health problems, but these links are either weak or inconsistent, and indeed, several of these same ingredients are commonly taken as remedies or preventive supplements to combat various common ailments.

For example, while there is some weak evidence that ginkgo is protective against pancreatitis[35,36], there are four low-quality "FDA reports" of pancreatitis in patients taking ginkgo[37]. Search of the presumably more reliable www.fda.gov site reveals no reports of this association.

While the health benefits and risks of caffeine are well known, none of these risks appear to be related to pancreatitis, and at least weak data suggest that caffeinated coffee may protect against alcoholic pancreatitis[38]. Similarly, no convincing association could be found between pancreatitis and other energy-drink ingredients, such as milk thistle and guarana. However, the FDA's Center for Food Safety and Applied Nutrition Adverse Event Reporting System has reported several associations between pancreatitis and the cocktails of various energy drinks, such as RedBull™, 5-h Energy Booster™, and Monster Energy™[18,19]. Nevertheless, the possibility remains that what we have observed in patient #4 is merely a coincidence between exacerbations of autonomous alcoholic pancreatitis and the consumption of energy drinks. Still the fact that the otherwise unexplained pancreatitis occurred in this single patient twice, both times immediately following consumption of the energy drinks, is striking.

In conclusion, there is insufficient direct evidence to support causality between pancreatitis and exposures to nerve stimulators and to energy drinks. However, the observations presented here, coupled with the rising use of the offending products, are cause for concern and warrant further study. Possibilities for such study include either cellular or animal models of pancreatitis using these potentially offending agents, analyses of large databases, and the establishment of an international registry.

COMMENTS

Case characteristics

Five cases of pancreatitis in four patients occurring after exposure to nerve stimulators or energy drinks.

Clinical diagnosis

Elevated lipase and/or imaging evidence of pancreatic inflammation.

Differential diagnosis

Pancreatitis could have been due to another cause not tested for, or missed, such as genetic polymorphisms or mutations.

Laboratory diagnosis

Elevated lipase, but normal IgG4 and triglycerides.

Imaging diagnosis

Computed tomography or magnetic resonance imaging showed evidence of pancreatic inflammation. It was not possible to rule out pancreas divisum in each and every case.

Pathological diagnosis

Pancreatic debridement in patient #3 yielded only necrotic and saponified tissue, as expected.

Treatment

The patients were treated with standard supportive care during their episodes of pancreatitis. Patient #3 required open pancreatic debridement and recovered well.

Related reports

The causes of idiopathic pancreatitis remain, of course, unknown, by definition. The authors know no other cases associating nerve stimulators and pancreatitis, and only very weak evidence exists associating some ingredients in energy drinks with pancreatitis.

Experiences and lessons

This case report presents several cases that suggest the possibility of new etiologies of pancreatitis. However, this observation should be interpreted with caution, as it is impossible to rule out every possible systemic, structural, and genetic cause of pancreatitis. In no way does this report prove any cause.

Peer-review

This is a very interesting manuscript from the clinical point of view. Both hypotheses are interesting.

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