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MINIREVIEWS

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MINIREVIEWS

Current therapies and novel approaches for biliary diseases

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Abstract

Chronic liver diseases that inevitably lead to hepatic fibrosis, cirrhosis and/or hepatocellular carcinoma have become a major cause of illness and death worldwide. Among them, cholangiopathies or cholestatic liver diseases comprise a large group of conditions in which injury is primarily focused on the biliary system. These include congenital diseases (such as biliary atresia and cystic fibrosis), acquired diseases (such as primary sclerosing cholangitis and primary biliary cirrhosis), and those that arise from secondary damage to the biliary tree from obstruction, cholangitis or ischaemia. These conditions are associated with a specific pattern of chronic liver injury centered on damaged bile ducts that drive the development of peribiliary fibrosis and, ultimately, biliary cirrhosis and liver failure. For most, there is no established medical therapy and, hence, these diseases remain one of the most important indications for liver transplantation. As a result, there is a major need to develop new therapies that can prevent the development of chronic biliary injury and fibrosis. This mini-review briefly discusses the pathophysiology of liver fibrosis and its progression to cirrhosis. We make a special emphasis on biliary fibrosis and current therapeutic options, such as angiotensin converting enzyme-2 (known as ACE2) over-expression in the diseased liver as a novel potential therapy to treat this condition.

Key words: Chronic liver disease; Biliary fibrosis; Current therapies for biliary fibrosis; Angiotensin converting enzyme-2; Gene therapy

Core tip: This mini-review focuses on the pathophysiology of chronic liver fibrosis, with a special emphasis on biliary fibrosis. We also attempted to provide information on current clinically available therapeutic options for biliary fibrosis and other potential therapeutic options that are in the preclinical stage of development, and discuss their

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advantages and disadvantages. In particular, work from the author's laboratory described in this review indicates that liver-specific over-expression of angiotensin converting enzyme-2 (known as ACE2) of the alternate renin angiotensin system dramatically reduces biliary fibrosis in mouse models of biliary disease. This suggests that ACE2 gene therapy has the potential to treat patients with chronic biliary fibrosis.

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INTRODUCTION

The prevalence of chronic liver diseases is rising worldwide, and approximately 1.7 million deaths are reported annually[1,2]. The aetiology of chronic liver diseases is multifactorial, and evidence from the literature indicates that these causative agents vary according to geographical location[3]. Major causes are chronic viral infections (*e.g.*, hepatitis B and C), excessive alcohol consumption, non-alcoholic fatty liver disease (NAFLD), inherited diseases (*e.g.*, Wilson's disease, biliary fibrosis) and primary sclerosing cholangitis (PSC), side effects of medications, toxic chemicals and idiopathic or cryptogenic causes[3,4]. Regardless of the aetiology, the events associated with pathogenesis and fibrogenic progression of chronic liver injury appear to share common intracellular pathways.

Hepatic fibrosis is the result of the wound-healing response of the liver to repeated injury. As a result, the balance between parenchymal cell regeneration and the wound healing response is shifted towards the wound healing response with impaired regenerative pathways over time, and hepatocytes are substituted with abundant extracellular matrix (ECM), eventually leading to accumulation of excess fibrotic scar tissue[5]. Cirrhosis is the end result of chronic liver diseases in which much of the hepatic parenchymal tissue is replaced by fibrous tissue, altering the liver function and distorting liver architecture with septae and nodule formation. This leads to alterations in blood flow with collateral formation, which ultimately results in cirrhosis and liver failure[4,6]. There are no established medical therapies for cirrhosis, and the ultimate therapy for this condition is liver transplantation, which is limited by the lack of donor livers and carries the risk of post-transplantation complications[4]. Thus, there remains a major need to identify potentially modifiable factors that exacerbate liver injury and fibrosis, and to develop therapies that can prevent or slow liver scarring.

Liver injuries are categorized into three major groups: cell-indiscriminate, cholestasis and hepatocyte-associated injuries. Mechanical trauma, ischemia and liver resection lead to cell-indiscriminate, whilst either mechanical or autoimmune bile duct injuries cause cholestasis. The major types of hepatocyte-associated injuries are either direct injuries (alcohol, drugs and hepatotropic infectious viruses, such as hepatitis B and C) or immune-mediated[2,7].

As injury persists, regardless of the initial cause, liver tissue responds by depositing ECM[8], which is known as the wound healing response. In addition, ECM synthesis is considered an effort of liver tissue to localize the injury by encapsulating the area of injury. Even though it is as an essential part of the wound healing process, the condition progresses to "liver fibrosis" once it is deregulated, which becomes an inefficient attempt at liver tissue remodelling[9,10]. Thus, liver fibrosis is mainly characterized by the excessive accumulation of ECM in the liver parenchyma that replaces functional hepatic tissue[11].

Interestingly, the microenvironment in the liver is an organized multidirectional interaction complex (cell-matrix-cell), which delivers the molecular signals crucial for normal liver homeostasis. In this process, each cell type in the liver, including hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs) and liver sinusoidal epithelial cells (LSECs), have their own roles to play while talking to each other, a process referred to as cellular "crosstalk"[12].

Activated HSCs are the main cell type that is responsible for ECM synthesis in the injured liver. In addition, they exert contractile and pro-inflammatory properties. During liver injury, HSC activation proceeds as a result of two major intercellular crosstalk pathways[12,13], which include capillarization[14,15] of LSECs and apoptosis of

hepatocytes[12,16]. It has been shown that KCs are also involved in cellular crosstalk during the process of fibrosis[12]. KCs are liver-resident macrophages that engulf apoptotic bodies arising from the apoptotic hepatocytes[12,17] and become activated. The activated KCs begin to express death ligands, such as Fas, TNF-α and TNF-related apoptosis-inducing ligand, that induce hepatocyte apoptosis in a feed-forward manner[17]. The activated KCs also release cytokines and reactive oxygen species through which they trigger the activation of HSCs in a paracrine manner[18].

Other than HSCs, there are myofibroblasts that are predominantly located around the portal tracts, particularly in cholestatic liver injuries. These myofibroblasts are derived from either bone marrow or small portal vessels as a response to cholestasis, and proliferate around biliary tracts[19]. In addition, the periportal myofibroblast cell population has been postulated to also derive from activated cholangiocytes[20]. These myofibroblast are also considered to play a role in collagen synthesis and perform a similar role to HSCs[19,21].

There is evidence that Mast cells are also involved in liver fibrosis as a response to injury (Figure 1). A Mast cell is a white blood cell in the circulation that contains histamine and heparin granules. It has been shown that Mast cell infiltration is evident during liver fibrosis in several rat models, including the bile duct-ligated model[22,23]. The infiltration of Mast cells into the liver during the progression of biliary fibrosis has also been described in multiple drug resistant gene 2 knockout (Mdr2-KO) mice, a mouse model of progressive biliary fibrosis. The presence of Mast cells increases local levels of histamine, which is a pro-fibrogenic and proliferative factor. It induces intrahepatic bile duct masses and ductular proliferation during fibrogenesis[24]. Transforming growth factor-beta 1 (TGF-β1), released by Mast cells, is a key pro-fibrotic cytokine that subsequently activates quiescent HSCs that produce ECM, leading to fibrosis[22,25,26]. In addition, Mast cells have the ability to induce the production of ECM components by overproduction of basement membrane, which induces fibroblast attachment, spreading and proliferation[22,27].

Cirrhosis is the end stage of liver fibrosis, and characterized by abnormal continuation of fibrogenesis and distortion of hepatic vasculature by neo-angiogenesis, a process involved in new sinusoid formation. In advanced stages of fibrogenesis, there is collective ECM synthesis from activated HSCs, myofibroblasts derived from bone marrow, portal fibroblasts and Mast cells that are closely associated with neo-angiogenesis and capillarization[10]. Cirrhosis is histologically characterised by vascularised fibrotic septa that link portal tracts and central veins, forming clusters of hepatocyte islands surrounded by fibrotic septa[28]. Thus, cirrhotic liver is characterized by diffuse fibrosis, regenerative nodules, altered lobular architecture and the establishment of intrahepatic vascular shunts between afferent vessels and efferent hepatic veins of the liver[10,29]. Some of the major clinical consequences of these distortions are the loss of liver function, development of portal hypertension (PHT), variceal bleeding and ascites, which can lead to renal failure and hepatic encephalopathy[28].

BILIARY DISEASES

Biliary diseases or cholangiopathies are a group of chronic liver diseases characterized by cholestasis and progressive biliary fibrosis that can lead to end stage liver failure. There are numerous aetiologies for these diseases. Two of the common cholangiopathies are immune disorders, primary biliary cholangitis/primary biliary cirrhosis (PBC)[30] and PSC. Infectious agents of bacterial, viral or fungal origin, vascular or ischemic causes (such as post-liver transplantation), hepatic artery stenosis, drugs/toxin and genetical abnormalities (such as cystic fibrosis) are also causes of cholangiopathies. There are also idiopathic cholangiopathies, including biliary atresia and idiopathic ductopenia. Many cholangiopathies, including PBC and drug-induced cholangiopathies, primarily affect small bile ducts. In contrast, diseases like PSC and cholangiocarcinoma affect both intra and extrahepatic large bile ducts[31]. Once bile flow is impaired, bile accumulates in the liver, causing primary damage to the biliary epithelium and eventually the liver parenchyma. A majority of cholangiopathies has similar features, including peri-portal inflammations that lead to liver fibrosis/cirrhosis. Given their progressive nature, most cholangiopathies cause substantial morbidity and mortality in patients and, thus, they are a major indication for liver transplantation[31,32].

PATHOGENESIS OF CHOLESTASIS AND BILIARY FIBROSIS

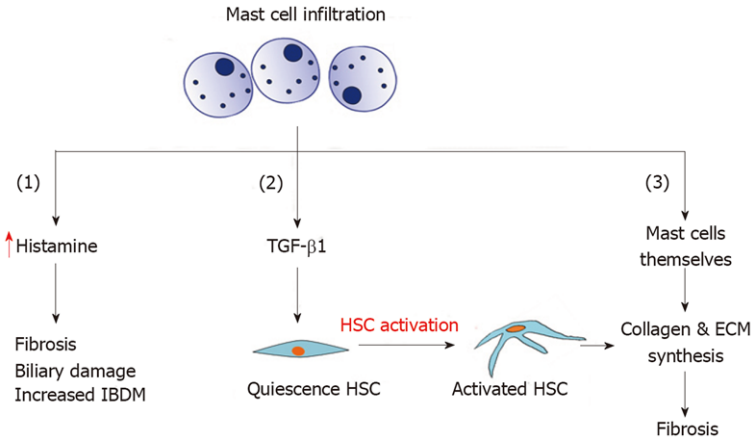

Figure 1 Mast cell infiltration and its role in biliary fibrosis. HSC: Hepatic stellate cell; ECM: Extracellular matrix; IBDM: Intrahepatic bile duct mass; TGF-β1: Transforming growth factor-beta 1.

Cholestasis is defined as a decrease in bile flow due to impaired secretion by hepatocytes or obstruction of bile flow. Obstruction of bile flow can occur due to intrahepatic or extrahepatic causes. Whilst intrahepatic bile duct obstruction and alterations in bile secretion by hepatocytes are considered as intrahepatic causes, obstruction in the extrahepatic bile duct is referred to as an extrahepatic cause of cholestasis[33]. Once bile flow is impaired, increased accumulation of bile within hepatocytes causes primary damage to biliary epithelium and eventually the liver parenchyma[34]. Many cholangiopathies, including PBC and drug-induced cholangiopathies, primarily affect small bile ducts. In contract, diseases like PSC and cholangiocarcinoma affect both intra and extrahepatic large bile ducts[31,35].

In chronic cholestatic liver injury, two major pathways are responsible for repairing the damaged cells and maintaining biliary homeostasis. The first is the proliferation of existing cholangiocytes (Figure 2A) of both small and large injured bile ducts, leading to the subsequent expansion of existing bile ducts. The second pathway is via activation of hepatic progenitor cells (HPCs) or oval cells[36,37], which differentiate into cholangiocytes, leading to the formation of new bile ducts, a condition referred to as "ductular reaction"[38] (Figure 2B). These newly formed ductules will eventually form a tubular network that restores the ductal mass in an attempt to prevent further liver injury and the leakage of bile acids into the liver parenchyma. In order to sustain newly formed tubules, a fibro-vascular stromal area is developed as a result of extensive cross-talk between hepatocytes, HSCs, LSECs and KCs[39]. On the other hand, ductular reaction is accompanied by continuous inflammatory signals resulting from key signalling molecules, such as TGF-β1, TNF-α and vascular endothelial growth factor, which then lead to liver fibrosis and later cirrhosis[36,40,41]. In late-stage cholangiopathies, ductopenia can occur that predominates over proliferation, leading to a state of vanishing bile ducts. The apoptosis rate of cholangiocytes becomes higher than that of the proliferation rate, and subsequently the cholangiocyte number is reduced, contributing to progressive portal fibrosis as seen in advanced cholangiopathies[31,42].

CURRENT TREATMENT OPTIONS FOR CHOLANGIOPATHIES

PBC and PSC are considered to be the most common cholangiopathies in humans. Both conditions lead to end stage liver failure, an indication for liver transplantation[43,44]. There has been a decrease in the number of liver transplantations for PBC in the United States and Europe after the clinical use of ursodeoxycholic acid (UDCA) in PBC patients[45]. Although it is the only Food and Drug Administration (FDA)-approved medical treatment for PBC, it has not been proven as a therapy for any other cholangiopathies[32,46]. PSC is the second most common cholangiopathy with no specific medical therapy, and current evidence shows that there is no reduction in the number of PSC patients listed for liver transplantation[47]. This indicates that there is no effective medical therapy to prevent PSC patient progression to cirrhosis[43,46]. Moreover, recurrence of PSC after liver transplantation emphasises the critical need for an effective medical therapy to treat this condition[48].

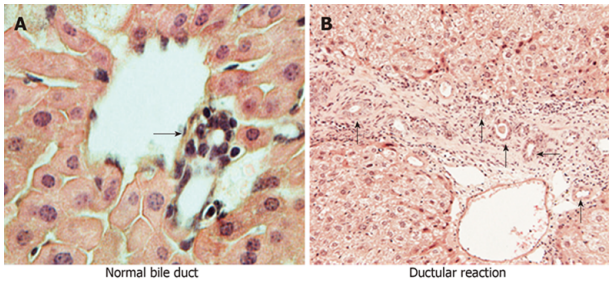

Figure 2 A bile duct consists of cholangiocytes in normal liver (A) and ductular reaction with reactive ductular cells in biliary diseases (B) (arrows indicate bile ducts).

The development of antifibrotic therapies holds promise in the treatment of liver fibrosis, including biliary diseases, irrespective of the cause of disease. They can be used to either prevent the formation of excessive ECM by inhibiting the activation of myofibroblastic cell populations or stimulate ECM degradation. However, the lack of availability of an effective antifibrotic therapy with minimal or no side effects is the main hurdle. As a result, liver transplantation has inevitably become the only option for patients with biliary fibrosis. An increased incidence of chronic liver disease, lack of donor organs, post-transplant complications and high cost associated with liver transplantation make the current situation worse. Therefore, there is a major need to develop and formulate specific, effective, safe and inexpensive medical treatments.

An exciting potential target to develop antifibrotic therapies is the local renin angiotensin system (RAS). In normal physiology, the RAS plays a pivotal role in blood pressure regulation and sodium and water homeostasis, as well as tissue remodelling after injury. It is now well-established that the RAS consists of two arms called the "classical arm" and the "alternate arm", which play counter-balancing roles. There is substantial evidence that angiotensin II (Ang II) is a main mediator in hepatic fibrosis, and circulating Ang II levels are elevated in patients with cirrhosis[49]. It has also been shown that the local RAS is also activated in the liver as a response to injury. Studies published by our laboratory and others have shown that once activated, there is increased expression of components of the classical RAS, including hepatic angiotensin converting enzyme (ACE) and Ang II type 1 receptor (AT1-R)[26,49]. Moreover, increased expression of classical RAS components is localized to the areas of active fibrogenesis, confirming that local RAS plays a pivotal role during hepatic fibrogenesis[22,50,51]. Consequently, attempts have been made to inhibit either the production of Ang II by ACE inhibitors (ACEi) or AT1-R activation by angiotensin receptor blockers (ARBs) in cirrhotic patients. This implies that ACEi and ARBs can be considered as potential pharmacological agents to block the effects of classical RAS to inhibit liver fibrosis[52]. Unfortunately, a major setback with this approach is that they produce off-target systemic side effects, including systemic hypotension and reduced renal perfusion.

Work from our laboratory has demonstrated that the alternate RAS, comprising ACE2 and the antifibrotic peptide angiotensin-(1-7) [Ang-(1-7)], is also activated in liver injury[22,53]. The alternate RAS is expected to counter the deleterious effects produced by activated classical RAS. In experimental cholestasis induced by bile duct ligation (BDL) in rats, the components of the classical RAS (including angiotensinogen, ACE and AT1-R) are upregulated at 1 wk post-BDL. However, the expression of components of the alternate RAS, such as ACE2, Ang-(1-7) and putative Ang-(1-7) receptor Mas (Mas-R), are delayed until the third week post-BDL. Upon activation, however, the expression of the alternate RAS parallels the changes of the classical RAS[53]. This, in turn, results in elevated circulating Ang-(1-7) levels[53]. These findings were corroborated with elevated levels of circulating Ang-(1-7) in patients with liver disease, confirming the activation of the alternate RAS during chronic liver injury[49,54].

Although inhibition of the components of the classical RAS has been extensively investigated in animal models of liver disease, there were only a few studies carried out to investigate the role of the alternate RAS in liver disease. Emerging evidence suggests that the alternate RAS is an attractive target for drug intervention in biliary fibrosis. One possible way of achieving a therapeutic outcome in biliary fibrosis would be to increase the level of antifibrotic peptide Ang-(1-7), the effector peptide of the alternate RAS, which opposes many of the deleterious effects of Ang II. Animal studies performed using BDL rats and cultured rat HSCs have confirmed that Ang-(1-7) peptide has the ability to reduce collagen secretion, leading to a profound improvement in hepatic fibrosis[49]. Moreover, the same study showed that the non-

peptide Mas-R agonist AVE0991 produced a significant decrease in α-SMA protein content and collagen production in rat HSCs. The findings that these effects were inhibited by Mas-R antagonist D-Ala^7-Ang-(1-7) (A779) suggest that the antifibrotic effects of Ang-(1-7) are mediated via Mas-R[49]. Moreover, an oral formulation of Ang-(1-7) has recently been developed where the peptide is encapsulated with oligosaccharide hydroxypropyl-cyclodextrin (HPβCD) to protect it from degradation by enzymes in the digestive system. This study showed that this oral Ang-(1-7) formulation was cardioprotective in rats with myocardial infarction[55].

Published work from the author's laboratory, however, suggested that the best way to achieve a therapeutic outcome in liver fibrosis is to target ACE2 of the alternate RAS. This is because enhanced expression and activity of liver ACE2 would be expected to provide dual benefits by increasing the degradation of profibrotic peptide Ang II with simultaneous generation of antifibrotic peptide Ang-(1-7). The evidence comes from animal studies showing that recombinant human ACE2 (rhACE2) is beneficial for the prevention of hypertension in cardiovascular disease[56] and the improvement of kidney function in diabetic nephropathy[57]. Recombinant hACE2 was shown to be well-tolerated by a group of healthy human volunteers in a phase 1 clinical trial without exerting any unwanted cardiovascular side effects[58]. However, randomized clinical trials with an adequate number of healthy individuals and patients assigned to receive rhACE2 treatment are yet to be undertaken. There is one study that reported therapeutic effects of rACE2 in experimental liver fibrosis, in which liver injury was induced by BDL or carbon tetrachloride (CCl_4) intoxication[59]. This study demonstrated that rACE2 reduced hepatic fibrosis in two animal models of liver disease[59]. Additionally, ACE2 gene knockout mice had elevated α-SMA protein and collagen content in the liver of CCl_4-induced cirrhotic animals compared with those of wild-type controls[59]. These findings suggest that ACE2 of the alternate RAS is a potential target for liver fibrosis.

A major disadvantage of systemic therapy is that the treatment will inevitably produce off-target effects, which in many cases are undesirable. Thus, there are several disadvantages with systemic administration of rACE2. This includes daily injections of ACE2[59], a procedure that is invasive in a clinical setting and an expensive approach[52]. Increased circulating ACE2 is highly likely to produce off-target effects, including an effect on blood pressure. To circumvent this problem, an ideal approach would be to increase tissue- or organ-specific ACE2 levels. Thus, organ-specific increased ACE2 activity would not only produce long-term organ-specific benefits, but would also minimize unwanted off-target effects.

ACE2 OVER-EXPRESSION IN THE LIVER

Viral vectors are effective and safe vehicles to introduce a transgene into specific tissues or organs. Of the viral vectors that have been used to date to increase the delivery of genes, adeno-associated viral (AAV) vectors appears to be the most safe and effective, and are widely used in Phase I-II clinical trials. The AAV vector has been shown to be efficient in the delivery of a transgene, and provides many advantages over other candidate viral vectors that include replicative defectiveness, non-pathogenicity, minimal immunogenicity and broad tissue tropism in both animal models and humans. The AAV system has become a popular tool for gene delivery with its ability to maintain long-term gene and protein expression following a single injection of the vector. This type of gene delivery system has been widely tested for inherited metabolic diseases[60]. It is significant that, for the first time, the FDA has approved a pioneering gene therapy protocol using an AAV vector for a rare form of childhood blindness in 2017, the first such treatment cleared in the United States for an inherited disease. Moreover, gene therapy using the AAV vector was approved in 2012 by the European Commission for the treatment of patients with lipoprotein lipase deficiency (LPLD)[61]. However, because LPL deficiency is an extremely rare genetic disorder in human and the treatment is expensive, UniQure, the company that produced AAV vector to treat LPLD, has not renewed its EU license in 2017.

In line with this, our group has developed a safe and effective therapeutic approach using a pseudotyped AAV vector, which uses the AAV2 genome and liver-specific AAV8 capsid (AAV2/8) to deliver murine ACE2 (AAV2/8-mACE2). This showed that a single intraperitoneal injection of rAAV2/8-mACE2 produces sustained elevation of liver ACE2 expression for up to 6 mo. The treatment was administered to three short-term mouse models with liver disease[62], which included liver disease induced by BDL (2-wk model), CCl_4 (8-wk model) and a methionine and choline deficient diet (8-wk model), representing cholestatic biliary fibrosis alcoholic liver fibrosis and NAFLD, respectively (Figure 3). AAV2/8-mACE2 therapy markedly

reduced hepatic fibrosis in all three models. More importantly, they further demonstrated that, in addition to sustained expression of liver ACE2 for up to 6 mo, ACE2 over-expression was absent in other major organs such as heart, lungs, brain, intestines and kidneys. Increased liver ACE2 expression and activity was accompanied by increased hepatic Ang-(1-7) levels with a concomitant decrease in hepatic Ang II levels[62].

We have now confirmed the effectiveness of this treatment strategy in Mdr2-KO mice, a long-term animal model with progressive hepato-biliary fibrosis. This model, which has been widely used for studies that investigated pathophysiology of biliary fibrosis, produces lesions that resemble those of human PSC[63-65]. Gene therapy using the AAV2/8-mACE2 vector was very effective in Mdr2-KO mice, showing 50% and 80% reduction in liver fibrosis in both established and advanced liver disease, respectively (Table 1).

SUMMARY

In clinical practice, although UDCA is the standard treatment for PBC, reports indicate that approximately 35%-40% of PBC patients do not achieve optimum responses to UDCA[30]. On the other hand, PSC among other cholangiopathies is a significant biliary disease, and studies in patients with PSC showed that whilst standard doses of UDCA are not effective, higher doses produce serious adverse events[66,67]. Thus, the lack of an effective pharmacotherapy for biliary diseases is often associated with the condition progressing to biliary cirrhosis, and bears the risk of developing into HCC or cholangiocarcinoma. Therefore, liver transplantation is considered as the only treatment option for patients with chronic cholangiopathies, such as end-stage PSC and PBC. However, the shortage of donor livers creates a large, unmet need to develop effective therapies for these conditions.

ACE2 gene therapy is a potential strategy to treat human biliary fibrosis by delivering ACE2 using human liver-specific novel vectors with high transduction efficiency[68]. Therefore, it is important to select an AAV vector specific for human hepatocytes with enhanced transduction efficiency[68]. Recent studies have shown that novel AAV vectors, such as AAV-LK03, AAV3B and AAVrh10, which have been identified by AAV DNA re-shuffling, transduce human primary hepatocytes at higher efficiency[68,69]. Since the FDA and EU have now endorsed human gene therapy, novel approaches of gene therapy research that employ human liver-specific AAV vectors will lead to the formulation of therapeutic gene therapy applications for human biliary fibrosis.

Table 1 mACE2-rAAV2/8 therapy increased hepatic ACE2 expression, resulting in a marked reduction in biliary fibrosis in a long-term model of chronic biliary fibrosis (Mdr2-KO mice)

Stage of the disease	Hepatic ACE2 expression, fold	Liver fibrosis reduction, %
Early: 3-6 mo	60	50%
Advanced: 7-9 mo	160	80%

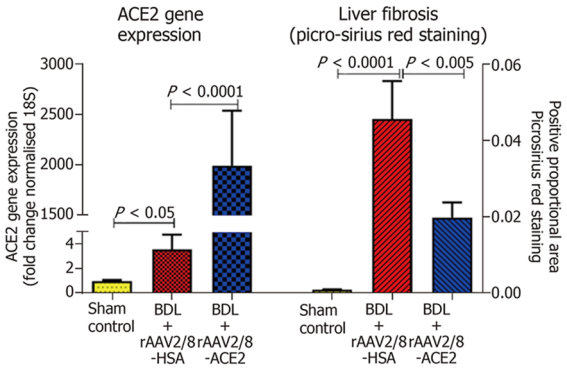

Figure 3 Hepatic *ACE2* gene expression and fibrosis in a short-term model of biliary fibrosis with rAAV2/8-ACE2 therapy. *ACE2* gene expression was significantly increased in ACE2-treated mice with biliary fibrosis compared to BDL mice injected with a control human serum albumin vector (rAAV2/8-HSA). rAAV2/8-ACE2 gene therapy markedly reduced the liver fibrosis in BDL mice compared to mice injected with rAAV2/8-HSA.

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EDITORIAL

Overview of studies of the vitamin D/vitamin D receptor system in the development of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. NAFLD is known to be associated with obesity, type 2 diabetes, metabolic syndrome and increased cardiovascular events: for these reasons, it is becoming a global public health problem and represents an important challenge in terms of prevention and treatment. The mechanisms behind the pathogenesis of NAFLD are multiple and have not yet been completely unraveled; consequently, at moment there are not effective treatments. In the past few years a large body of evidence has been assembled that attributes an important role in hepatic aberrant fat accumulation, inflammation and fibrosis, to the vitamin D/vitamin D receptor (VD/VDR) axis, showing a strong association between hypovitaminosis D and the diagnosis of NAFLD. However, the data currently available, including clinical trials with VD supplementation, still provides a contrasting picture. The purpose of this editorial is to provide an overview of recent advances in the pathogenesis of NAFLD in relation to VD/VDR. Based on recent data from literature, we focused in particular on the hypothesis that VDR itself, independently from its traditional ligand VD, may have a crucial function in promoting hepatic fat accumulation. This might also offer new possibilities for future innovative therapeutic approaches in the management of NAFLD.

Key words: Vitamin D; Vitamin D receptor; Non-alcoholic fatty liver disease; Type 2 diabetes

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Core tip: In the last years, many evidences attribute to the vitamin D/vitamin D Receptor axis an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The purpose of this editorial is to provide an overview of recent advances in the pathogenesis of NAFLD in relation to vitamin D/vitamin D receptor (VD/VDR). We focused in particular on the hypothesis that VDR itself, independently from its traditional ligand VD, may play a crucial function in promoting hepatic fat accumulation, also offering new possibilities for innovative therapeutic approaches in the management of NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common chronic liver disease worldwide[1]. Recent epidemiologic studies report that the prevalence of NAFLD is increasing, starting from the currently estimated 25% in the general population[2,3], and rising dramatically in obese individuals[4], in subjects with type 2 diabetes (T2D)[5] and those with metabolic syndrome[6]. NAFLD is becoming a global public health problem[7]: In many countries the number of patients affected by the disease is rapidly growing, so that in the last years the disease has reached epidemic proportions. Moreover, several studies have shown increased cardiovascular events in NAFLD patients and demonstrated that NAFLD is an independent risk factor for cardiovascular mortality[8-10].

VITAMIN D AND NAFLD

In spite of the alarming prevalence and the clinical implications of NAFLD, the mechanisms underlying its development and progression are still not fully understood, and currently there are no effective treatments. Over the years many different pathophysiological theories have been put forward, leading to the most widely accepted hypothesis, "multiple parallel hits"[11]. According to this model the steps conducive to hepatic fat accumulation, inflammation and fibrosis are orchestrated by a delicate interplay of factors[11], and in this context the role of the vitamin D/vitamin D receptor (VD/VDR) axis has become an active area of research. Indeed, apart from its central role in bone and mineral homeostasis, VD is a molecule that exerts various effects on a number of biological systems; active VD in particular has been shown to regulate the immune system and to modulate insulin sensitivity in experimental models of metabolic diseases[12-14].

Numerous studies have demonstrated that low VD circulating levels are associated with obesity[15], metabolic syndrome[16-19], and T2D[20-22]. Investigations conducted in several adult populations also showed a strong association between hypovitaminosis D and the diagnosis of NAFLD[23-30]. This association was also confirmed in children, in which low VD levels were found to correlate with the histological severity of NAFLD independently from metabolic characteristics[31,32].

Data from animal studies further support the notion that the impairment of VD balance plays a role in the development of NAFLD. Roth and colleagues showed that in obese rats the lack of VD intake allowed the onset and progression of NAFLD, which was evaluated through liver histology demonstrating a higher NAFLD activity score and increased lobular inflammation[33]. Likewise, under experimental conditions, VD has been shown to have an anti-inflammatory effect, accompanied by a significant inhibition of the hepatic expression of pro-fibrotic mediators, such as platelet-derived growth factor and transforming growth factor. The anti-inflammatory effect was also demonstrated by the suppression of the production of collagen, α-smooth muscle actin and tissue inhibitors of metalloproteinase-1 β[34-37]. In addition, in a study conducted on mice with nonalcoholic steatohepatitis (NASH), phototherapy reduced hepatocyte inflammation and fibrosis and improved insulin resistance by increasing the serum active form of VD[38].

On the basis of these evidences and of both experimental and epidemiological data, VD has attracted the interest for a potential therapeutic option during NAFLD. However, up until now results from randomized clinical trials have failed to demonstrate the efficacy of VD supplementation in improving either fatty liver content, or the histological parameters of inflammation and fibrosis, or transaminases in the course of NAFLD and NASH[39-45].

The clinical significance of VD in NAFLD is thus still controversial. A critical examination of the results from trials conducted so far may provide reasonable grounds for conducting further appropriately designed investigations (for example, personalized supplementation regimes in relation to VD levels at baseline and stage of liver damage, higher VD supplementation doses, longer periods of supplementation) before reaching any final conclusions on this topic. However, at present it is not possible to recognize which real benefits can be obtained from restoring optimal VD values in the case of chronic hepatic damage as a result of NAFLD.

ROLE OF VDR

In addition to the question of vitamin D, the role of VDR *per se* has been investigated in metabolic diseases, focusing in particular on its effect/expression in insulin sensitive tissues and organs, such as adipose tissue and the liver. In 2012, Barchetta *et al*[46] demonstrated for the first time in humans the expression of VDR in different hepatic cell types and reduced VDR expression in the hepatic cells of patients with NASH. Since that time many studies have shown that in the liver VDR regulates necro-inflammation and fibrosis[47-50]. Moreover, Arai *et al*[51] recently demonstrated that, in patients with biopsy-proven NAFLD, polymorphisms of the VDR gene are associated with the severity of liver fibrosis.

Interestingly the data showed that not only VD, but also secondary hydrophobic bile acids, such as lithocholic acid, activate VDR in human hepatocytes[52,53]. Bozic *et al*[50] demonstrated that in animal models, the development of liver steatosis was blunted in the presence of VDR deletion. Notably, data obtained in mice exposed to a high fat diet showed an early induction of hepatic VDR expression in the presence of a fatty liver, followed by a trend towards VDR reduction in the long term, whereupon more severe inflammation and fibrosis occurred[50]. In that same research, an expression analysis of genes related to lipid metabolism in mouse livers indicated that VDR might exert a pro-steatotic activity in the hepatocytes as results of both the activation lipogenic pathways and the inhibition of fat oxidation. Moreover, García-Monzón *et al*[54] very recently demonstrated that hepatic angiopoietin-like protein 8 (ANGPTL8) expression is increased upon VDR activation. It is known that ANGPTL8 is a key regulator of triglycerides metabolism and that higher circulating ANGPTL8 levels are associated with the presence of NAFLD[55-57]. These data suggest that VDR induction is more prominent in simple steatosis than in advanced liver damage, which is likely to indicate that VDR is induced at the early stages of the disease and does not require liver injury or fibrosis to have become established.

The overall data appear to support the hypothesis that, in the course of metabolic diseases, VDR itself, independently from its traditional ligand VD, may have a crucial function in promoting hepatic fat accumulation. Further studies should be oriented in this direction with a view to fully understanding the processes behind hepatic VDR activation and evaluating its role as a new target for innovative therapeutic approaches to the early management of NAFLD.

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MINIREVIEWS

Immune checkpoint inhibitor-induced diarrhea/colitis: Endoscopic and pathologic findings

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Abstract

The indications of immune checkpoint inhibitors (ICPIs) for cancer treatment have rapidly expanded, and their use is increasing in clinical settings worldwide. Despite the considerable clinical benefits of ICPIs, frequent immune-related adverse events (irAEs) have become nonnegligible concerns. Among irAEs, ICPI-induced colitis/diarrhea is frequent and recognized not only by oncologists but also by gastroenterologists or endoscopists. The endoscopic findings show similarity to those of inflammatory bowel disease to a certain extent, particularly ulcerative colitis, but do not seem to be identical. The pathological findings of ICPI-induced colitis may vary among drug classes. They show acute or chronic inflammation, but it may depend on the time of colitis suggested by colonoscopy, including biopsy or treatment intervention. In the case of chronic inflammation determined by biopsy, the endoscopy findings may overlap with those of inflammatory bowel disease. Here, we provide a comprehensive review of ICPI-induced colitis based on clinical, endoscopic and pathologic findings.

Key words: Immune checkpoint inhibitor; Colitis; Diarrhea; Endoscopic; Pathologic

Core tip: Immune checkpoint inhibitor (ICPI)-induced colitis/diarrhea is frequent and recognized not only by oncologists but also by gastroenterologists or endoscopists. The endoscopic findings resemble those of inflammatory bowel disease to a certain extent, particularly ulcerative colitis, but are not identical. The pathological findings of ICPI-induced colitis may vary among drug classes. The findings show acute or chronic phases

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but may depend on the diagnostic timing or treatment intervention. Colonoscopy with biopsy is necessary to confirm ICPI-induced colitis, and early evaluation may avoid exacerbating or prolonging colitis due to treatment resistance.

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INTRODUCTION

In 1992, Ishida *et al*[1] identified a protein on activated T lymphocytes called programmed cell death protein 1 (PD-1), a key player in tumor immunology. In 1996, Leach *et al*[2] identified a protein called cytotoxic T-lymphocyte antigen-4 (CTLA-4), another major blocking pathway for the human immune system that was similar to PD-1. Since then, their discoveries have led to the development of immune checkpoint inhibitors (ICPIs) as anticancer drugs and have brought about a major revolution in cancer treatment strategy. Both CTLA-4 and PD-1 deliver negative signals to T-cell-mediated excessive immune activation, known as checkpoints, and ICPIs disrupt the signals mediated by CTLA-4 and PD-1 to prevent T cells from blocking pathways. By inhibiting immune checkpoints, activation of T cells is maintained, thereby helping cancer cells to induce cytotoxic T cell-mediated death. In 2018, Professor Honjo and Professor Allison won the Nobel prize in Physiology or Medicine for their work.

Presently, there are six ICPIs available and approved by the United States Food and Drug Administration for different cancers. Despite the significant clinical benefits of ICPIs, frequent immune-related adverse events (irAEs) in the skin, endocrine organs, gastrointestinal (GI) tract, liver, and lungs and in the musculoskeletal, renal, nervous, hematologic, cardiovascular, and ocular systems have become nonnegligible concerns. Most irAEs have a delayed onset and prolonged duration compared with those from chemotherapy[3]. The incidence of irAEs appears to be similar across tumor types[4]. Among irAEs, ICPI-induced colitis/diarrhea is frequent and recognized not only by oncologists but also by gastroenterologists or endoscopists. In this review, we provide a comprehensive review of ICPI-induced colitis based on clinical, endoscopic and pathologic findings.

ONSET TIMING OF ICPI-INDUCED DIARRHEA/COLITIS

ICPI-induced diarrhea occurs after an average of three infusions[5], although it can occur immediately after the first infusion. Recent reports suggest that the onset timing of ICPI-induced diarrhea/colitis may differ by ICPI type. ICPI-induced diarrhea/colitis induced by ipilimumab (anti-CTLA-4) usually occurs 6 to 7 wk after the initiation of ipilimumab[6]. The median time from last the ipilimumab treatment to diarrhea onset is 11-14 d (range 0-59 d)[7,8]. On the other hand, Wang *et al*[9] reported that 3.2% of patients (30/973) receiving anti-PD-1 developed ICPI-induced colitis at a median of 25.4 wk (range 0.6-120 wk). ICPI-induced diarrhea/colitis induced by anti-PD-1 seems to occur later than that induced by anti-CTLA-4. After the combined use of ipilimumab and nivolumab or pembrolizumab, 24.4% of patients (79/324) developed ICPI-induced diarrhea/colitis significantly earlier, at a median of 7.2 wk (range 0.7-51 wk)[9]. Because the ranges of its onset timing are widely distributed, it is difficult to predict the development of ICPI-induced diarrhea/colitis. In addition, it may be influenced by other drugs, including NSAIDs, antibiotics, or previous anticancer drugs. Moreover, it seems difficult to predict the development of colitis before patients have symptoms[10]. We should keep in mind that ICPI-related colitis can occur at any point, even after discontinuation of ICPIs.

LOCATION

Geukes Foppen *et al*[11] reported total colonoscopy in 62 of 92 patients (67%) suspected of ICPI-induced colitis. Of these patients, 68% showed pancolitis (> 3 affected

segments), and the ascending colon had more severe colitis than the descending colon. In cases where a total colonoscopy was not performed, patients with colitis in the ascending colon can be underestimated by sigmoidoscopy alone. Abdominal computed tomography (CT) findings may be useful not only to evaluate perforation, obstruction, and toxic megacolon but also to evaluate inflamed lesions due to ICPIs. The common CT findings of 16 patients treated with ipilimumab showed that 75% of patients had diffuse colitis patterns, and 25% had segmental colitis[12]. CT was not sufficient to diagnose colitis when using endoscopic evaluation as the gold standard because it has a high false-negative rate and low sensitivity[13]. In contrast, Garcia-Neuer *et al*[14] reported that CT was useful for predicting ICPI-induced colitis with a positive predictive value of 96% and a negative likelihood ratio of 0.2 in 34 diarrhea patients who underwent both CT and colonoscopy with biopsy. Early sigmoidoscopy without bowel preparation has merit to assess ICPI-induced colitis because it can be performed more easily and earlier than total colonoscopy. Therefore, the combined use of sigmoidoscopy and CT may be useful to evaluate ICPI-induced colitis at an earlier stage.

ENDOSCOPIC EVALUATION AND FINDINGS

There are several reports about the endoscopic findings of ICPI-induced colitis. Wang *et al*[13] observed that endoscopic inflammatory findings were found in more than 80% of patients with ICPI-induced diarrhea/colitis. Common endoscopic inflammation findings are reported as exudates, loss of vascular pattern, granular or edematous mucosa, patch or diffuse erythema, aphtha and ulcerations (Figure 1)[15,16]. Most of the inflammatory changes, including pathological changes, are dominantly more diffuse than patchy[10], but patchy distribution was endoscopically observed in half of the patients with diarrhea[17]. These endoscopic findings resemble those of inflammatory bowel disease (IBD) to a certain extent, particularly with ulcerative colitis (UC)[16,18], but sometimes look different from a UC-like pattern (Table 1).

Wang *et al*[13] reported in 53 patients with diarrhea, clinical symptoms did not always correlate with other endoscopic findings except for the presence of ulceration, which had a strong relationship with higher colitis. Similarly, another retrospective study showed that there was no significant correlation between diarrhea/colitis symptoms and endoscopic findings in 92 patients who developed diarrhea.

They also reported that pancolitis and the presence of ulceration are indicators for steroid-refractory colitis[11]. Geukes Foppen *et al*[11] reported that the Mayo score was associated with the presence of ulceration. Abu-Sbeih *et al*[19] categorized endoscopic findings as low-risk and high-risk for steroid-responsiveness. High-risk findings included either ulcers deeper than 2 mm and/or larger than 1 cm in surface area or endoscopically extensive colitis from the proximal colon to the splenic flexure. These patients require frequent use of infliximab or vedolizumab and more frequent and longer hospital stays than non-high-risk patients[19]. They also reported that timely early colonoscopy decreased the duration of steroid treatment[19]. If the colonoscopy shows normal mucosal findings, we are not always able to exclude the presence of ICPI-induced colitis, as cases of isolated ileitis[20] or enteritis without colitis[21] can also occur. We can also rule out microscopic colitis or other infectious diseases such as *Clostridioides difficile* or cytomegalovirus[7]. Therefore, early colonoscopy with mucosal biopsy from colorectal and ileum-end mucosa is necessary not only to evaluate the severity and distribution of colitis[11] but also to ensure shorter and less intense treatment[19].

PATHOLOGY

The histologic features of ICPI-associated colitis may vary among drug classes, *i.e.*, CTLA-4 inhibitors and PD-1/PDL1 inhibitors. Although they are nonspecific, some findings can be helpful clues to diagnose and speculate about the class of inhibitors. On the other hand, there is significant overlap between ICPI-associated colitis and other types of colitis, making the differential diagnosis difficult.

The histologic findings of CTLA-4-associated colitis are relatively consistent across most studies. The previously reported histologic features of CTLA-4 associated colitis are similar to those of autoimmune colitis[22]. They include lamina propria expansion due to dense lymphoplasmacytic infiltrate, increased intraepithelial lymphocytosis, and apoptosis in the crypts. Neutrophilic cryptitis and crypt abscess are also found. At times, there is prominent eosinophilia in the lamina propria. Although dense lymphoplasmacytic lamina propria expansion is reminiscent of other mimics, the lack

Table 1 Summary of endoscopic and pathological findings of immune-related diarrhea and colitis

Endoscopic and pathological findings of immune-related diarrhea and colitis	
Endoscopic findings	
Endoscopic features	(1) Exudates; (2) loss of vascular pattern; (3) granular or edematous mucosa; (4) patch or diffuse erythema; (5) aphtha; (6) ulceration
Inflammatory distribution	(1) Diffuse; (2) patchy (dominantly more diffuse than patchy)
Risk factors for steroid-refractory colitis	(1) Extensively inflamed area (*e.g.*, pancolitis); (2) deeper ulceration
Pathological findings	
Anti-CTLA-4 associated colitis	Like autoimmune colitis: (1) lamina propria expansion due to dense lymphoplasmacytic infiltrate; (2) increased intraepithelial lymphocytosis; (3) apoptosis in the crypts; (4) neutrophilic cryptitis and crypt abscess; (5) occasional prominent eosinophilia in the lamina propria; (6) the lack of findings of basal plasmacytosis, crypt distortion, or granulomas
Anti-PD1/anti-PDL1-associated colitis	(1) Expansion of lamina propria by lymphoplasmacytic infiltrate; (2) the increase in intraepithelial neutrophils and neutrophilic crypt abscess; (3) crypt distortion; (4) increased crypt cell apoptosis

CTLA-4: Cytotoxic T-lymphocyte antigen-4; PD1: Programmed cell death protein 1; PDL1: Programmed cell death receptor ligand 1.

of findings of basal plasmacytosis, crypt distortion, or granulomas can help the differentiation.

The most common findings of anti-PD1/anti-PDL1-associated colitis are the expansion of the lamina propria by lymphoplasmacytic infiltrate and features of active colitis[23-27]. The latter are characterized by an increase in intraepithelial neutrophils and neutrophilic crypt abscess (Figure 2A). Other findings include crypt distortion, increased crypt cell apoptosis, features of ischemic colitis, and collagenous colitis (Figure 2B). Although, in the study by Gonzalez *et al*[26], there were no cases with increased intraepithelial lymphocytosis commonly observed in CTLA-4-associated colitis, Chen *et al*[23] and Bavi *et al*[27] described features of lymphocytic colitis in a minority of their cases with anti-PD1/anti-PDL1. In the latter studies, a PD-1 inhibitor and CLTA-4 inhibitor were prescribed for their patient population either in combination or sequentially. Therefore, it is unlikely that this finding is related to PD-1 inhibition alone.

As mentioned, the histologic features of ICPI-associated colitis are nonspecific and can mimic other type of colitis, including infectious colitis, IBD, graft versus host disease (GVHD), and other drug-induced colitis. Although infectious colitis typically shows features of active colitis, increased apoptosis and crypt atrophy/dropout are not typical features[28]. ICPI-associated colitis lacks the features of chronicity that characterize IBD[29]. The lamina propria expansion by lymphoplasmacytic infiltrate can discriminate from GVHD although increased crypt apoptosis is the *sine qua non*e of the diagnosis of GVHD[30]. Despite the histopathological differential diagnostic points, clinical correlation and medical history are indispensable for discrimination between ICPI-associated colitis and mimics (Table 1).

MORBIDITY ASSOCIATED WITH ICPI-INDUCED DIARRHEA/COLITIS AND TREATMENT

IrAEs involving the GI tract range from mild to severe events[31] and are well reported for anti-CTLA4 but less well reported for anti-PD-1 and anti-PD-L1 and for combined anti-CTLA4 plus anti-PD-1. Most clinical trials distinguish diarrhea from colitis even though they overlap in most practical cases. Diarrhea is evaluated based on an increase in stool per day or ostomy output. Colitis is evaluated based on clinical symptoms (abdominal pain, mucus or blood in stool) or diagnostic observations based on radiographic and/or colonoscopy findings. The severity is usually classified based on the Common Terminology Criteria for Adverse Events[32] (Table 2).

Moderate to severe ICPI-related colitis may lead to severe deterioration in organ function and quality of life and life-threatening events. Diarrhea and colitis occurred in 8% to 22% of patients treated with anti-CTLA4[15]. A recent systemic review reported that 613 fatal ICPI toxic events were fond from 2009 through January 2018 searched by Vigilyze, which included 135 anti–CTLA-4 deaths and 32 combination anti-CTLA-4 plus anti-PD-1 deaths from colitis (27%)[33]. Colonic perforation was reported to occur in 1-5% of melanoma patients treated with ipilimumab (anti-CTLA-4)[7,15,34], and 0.6% of patients treated with ipilimumab died due to ICPI-induced

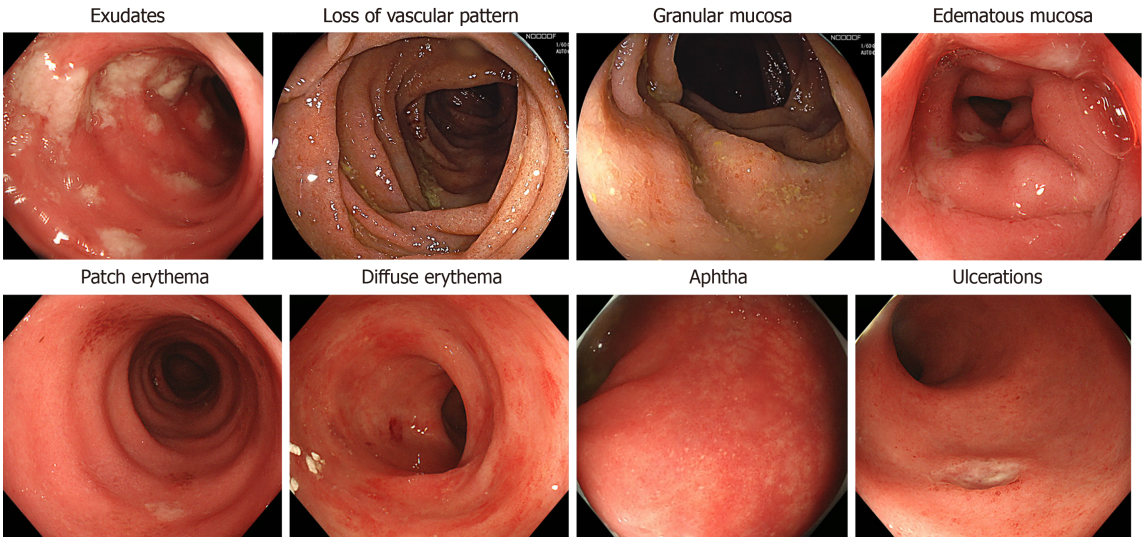

Figure 1 Endoscopic findings caused by an immune checkpoint inhibitor.

colitis[35].

Anti-CTLA4-related colitis is reportedly associated with mouth ulcers, anal lesions and extraintestinal irAEs[17]. A recent meta-analysis of 34 studies that included 8863 patients in clinical trials revealed that, for anti-CTLA4 alone (ipilimumab), all grades of colitis occurred in 9.1% (95% confidence interval (CI), 6.6%-12.5%) of participants, grade 3/4 colitis occurred in 6.8% (95%CI: 5.3%-8.6%) of participants, and grade 3/4 diarrhea occurred in 7.9% (95%CI: 5.5%-11.4%) of participants. Similarly, for anti-PD-1 alone (nivolumab or pembrolizumab), the rates were 1.4% (95%CI: 1.1%-1.8%), 0.9% (95%CI: 0.7%-1.3%), and 1.3% (95%CI: 1.0%-1.7%), respectively. For anti-PD-L1 alone (atezolizumab), the rates were 1.0% (95%CI: 0.4%-2.2%), 0.6% (95%CI: 0.2%-1.6%), and 0.3% (95%CI: 0.1%-1.1%), respectively[36]. For anti-CTLA4 (Ipilimumab) plus anti-PD-1 (nivolumab), the rates were 13.6% (95%CI: 7.7%-22.9%), 9.4% (95%CI: 4.8%-117.4%), and 9.2% (95%CI: 6.8%-12.3%), respectively. ICPI-induced diarrhea/colitis induced by anti-CTLA-4 can develop more often and more severely than ICPI-induced diarrhea/colitis induced by anti-PD-1. Combined anti-CTLA4 plus anti-PD-1 treatment is also more strongly associated with diarrhea/colitis than single-drug treatment[36]. Ipilimumab is commonly used at either 10 mg/kg or 3 mg/kg. There were similar rates of severe colitis at these doses, but severe diarrhea was more frequent at a dose of 10 mg/kg than at 3 mg/kg[36]. Recently, Marthey *et al*[17] showed that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) was associated with an increased risk of ICPI-induced colitis induced by CTLA-4 (2/38, 5% *vs* 11/35, 31%, $P = 0.003$). Therefore, the use of NSAIDs may affect the incidence of ICPI-induced diarrhea/colitis. Table 3 shows a summary of the incidence of immune-related diarrhea or colitis based on representative clinical trials.

In the case of grade 1 diarrhea/colitis, antidiarrheal drugs and/or oral hydration with electrolyte substitution can be initiated. In cases of persistent or grade 2 or higher diarrhea or rectal bleeding, it is necessary to confirm colitis or to rule out GI infection by testing for stool leukocytes, stool cultures, IBD, or tumor-related GI symptoms. In particular, *Clostridioides difficile* toxin and/or antigen test, cytomegalovirus DNA polymerase chain reaction, and tests for stool ova and parasites should be carried out in every patient with diarrhea treated with ICPIs. Sigmoidoscopy or colonoscopy combined with mucosal biopsy needs to be performed to evaluate the presence of colitis and to rule out GI metastasis because it is not uncommon in lung cancer or melanoma. If ICPI-induced colitis is diagnosed, an oral steroid is recommended. In the case of grade 3/4 diarrhea/colitis or persistent symptoms after oral steroids for several days, changing the treatment to intravenous steroids should be considered, and an infusion solution with electrolytes should be given. If patients respond to intravenous steroids within several days, they should be switched to oral steroids and tapered. However, if they fail to respond to steroid infusion, treatment with anti-TNF-α should be considered[15,37]. Recently, a case series reported that vedolizumab was a safer and more theoretic alternative than anti-TNF in patients with steroid-dependent or partially refractory ICPI-induced enterocolitis[38]. In the near future, vedolizumab may be effective and safe because it inhibits the migration of mucosal-associated T lymphocytes without inducing immune suppression and does not show an increased risk of serious infections in patients with UC or Crohn's disease[39,40].

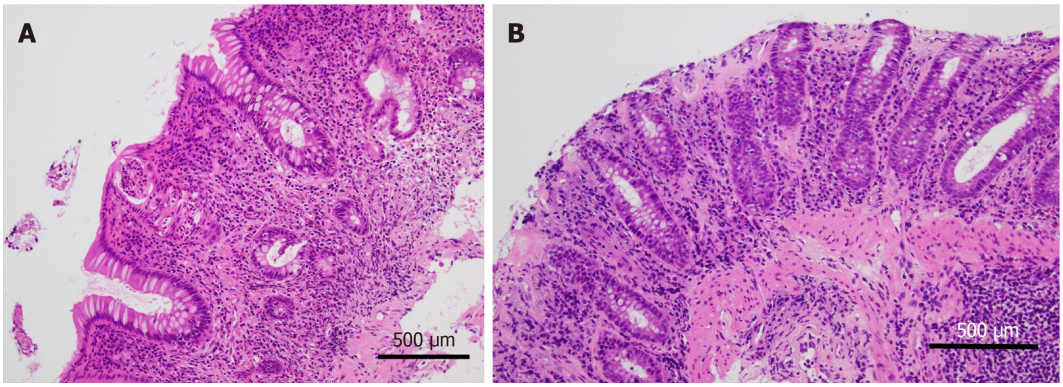

Figure 2 Programmed cell death protein 1 inhibitor-associated colitis. A: This colon biopsy reveals lamina propria expansion by lymphoplasmacytic infiltrate. Crypt distortion, crypt abscess, and cryptitis are prominent in the mucosa. In the stroma, a significantly increased eosinophilic infiltrate is observed; B: In another case of immune checkpoint inhibitors-related colitic mucosa, a subluminal collagen band thickening is prominent as observed in collagenous colitis. (Hematoxylin and eosin original magnification × 20, a scale bar represents 500 μm).

CONCLUSION

The combination of endoscopic and pathological findings may help diagnose ICPI-induced colitis as well as exclude infectious colitis, including *Clostridioides difficile* or cytomegalovirus, ischemic colitis, other drug-induced colitis, or segmental diverticular colitis. However, there are no specific findings because the endoscopic and pathological findings can depend on the time of colitis proven by biopsy or treatment intervention. In cases of persistent or grade 2 or higher diarrhea or rectal bleeding, colonoscopy evaluation is necessary to confirm ICPI-induced colitis and to rule out other diseases. Early evaluation and intervention may avoid exacerbating or prolonging colitis.

Table 2 Definition of diarrhea and colitis based on Common Terminology Criteria for Adverse Events v5.0[32]

CTCAE Term	Definition	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	CTCAE v5.0 Change
Diarrhea	A disorder characterized by an increase in frequency and/or loose or watery bowel movements	Increase of < 4 stools per day over baseline; mild increase in ostomy output compared to baseline	Increase of 4-6 stools per day over baseline; moderate increase in ostomy output compared to baseline; limiting instrumental ADL	Increase of ≥ 7 stools per day over baseline; hospitalization indicated; severe increase in ostomy output compared to baseline; limiting self-care ADL	Life-threatening consequences; urgent intervention indicated	Death	Clarification: Grade 2, 3, Definition
Colitis	A disorder characterized by inflammation of the colon	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Abdominal pain; mucus or blood in stool	Severe abdominal pain; peritoneal signs	Life-threatening consequences; urgent intervention indicated	Death	Addition: Navigational note; Clarification: Grade 3

ADL: Activities of daily living; CTCAE: Common Terminology Criteria for Adverse Events.

Table 3 Summary of incidence of immune-related diarrhea and colitis

ICPI	Target	Author	Year	Plus other drugs	*n*	Cancer type	Any gradediarrhea /colitis, *n* (%)	Grade 3-5diarrhea/colitis, *n* (%)
Nivolumab	PD-1	Topalian *et al*[41]	2012	None	296	Solid cancer	33 (11)/ND	3 (1)/ND
		Weber *et al*[42]	2013	None ipilimumab-naive	34	Melanoma	13 (38.2)/0 (0)	Not observed[1]
				Ipilimumab-refractory	56		11 (19.6)/0 (0)	
		Weber *et al*[43]	2015	None	268	Melanoma[2]	30 (11.2)/ND	1 (0.4)/ND
		Larkin *et al*[44]	2015	None	315	Melanoma	60 (19.2)/4 (1.3)	7 (2.2)/2 (0.6)
		Ferris *et al*[45]	2016	None	236	SCCHN	16 (6.8)/0 (0)	0 (0)/0 (0)
		Kang *et al*[46]	2017	None	330	GC/GEJC	23 (7)/2 (1)	2 (1)/1 (< 1)
Pembrolizumab	PD-1	Hamid *et al*[47]	2013	None	135	Melanoma	27 (20)	1(1)
		Garon *et al*[48]	2015	None	495	NSCLC	40 (8.1)/ND	3 (0.6)/ND
		Ribas *et al*[49]	2015	None	361	Melanoma[2]	32 (8.9)/5 (1.4)	2 (0.6)/2 (0.6)
		Herbst *et al*[50]	2016	None	690	NSCLC	46 (6.7)/6 (0.9)	2 (0.3)/4 (0.6)
		Ribas *et al*[51]	2016	None	655	Melanoma	115 (18)/11(2)	6 (1)/7 (1.1)
		Mok *et al*[52]	2019	None	636	NSCLC	34 (5)/7 (1)	5 (< 1)/4 (< 1)
Ipilimumab	CTLA-4	Weber *et al*[53]	2008	None	88	Melanoma	ND	5 (5.6)/4 (4.5)
		Weber *et al*[54]	2009	None	57	Melanoma	20 (35)/ND	10 (18)/ND
				budesonide	58		19 (33)/ND	8 (14)/ND
		Wolchok *et al*[55]	2010	None	214	Melanoma	58 (27)/ND	11(5.1)/ND
		Hodi *et al*[56]	2010	None	131	Melanoma	43 (32.8)/10 (7.6)	7 (5.3)/7 (5.3)
				gp100	380		146 (38.4)/20 (5.3)[3]	17 (4.5)/12(3.2)[3]
		Robert *et al*[57]	2011	Dacarbazine	247	Melanoma	81 (32.8)/11 (4.5)	10 (4.0)/5 (2.0)
		Margolin *et al*[58]	2012	None	72	Melanoma	30 (42)/ND	6 (8.3)/ND
		Kwon *et al*[59]	2014	None	399	Prostate cancer	199 (51)/27 (7)	64 (16)/18 (5)
		Larkin *et al*[44]	2015	None	311	Melanoma	103 (33.1)/36 (11.6)	19 (6.1)/27 (8.7)
		Eggermont *et al*[35]	2016	None	471	Melanoma	194 (41.2)/73 (15.5)	46 (9.8)/39 (8.2)

Ipilimumab plus nivolumab	CTLA4 and PD1	Wolchok *et al*[60]	2013	None	53	Melanoma	18 (34.0)/5 (9)	3 (6)/2 (4)
		Larkin *et al*[44]	2015	None	315	Melanoma	138 (44.1)/37 (11.8)	29 (9.3)/24 (7.7)
		Schadendorf *et al*[61]	2017	None	407	Melanoma	30 (7.4)/40 (9.8)	25 (6.1)/32 (7.9)
		Wolchok *et al*[62]	2017	None	313	Melanoma	142 (45)/40 (13)	29 (9)/26 (8)
		Hellmann *et al*[63]	2017	None	77	NSCLC	16 (21)/4 (5.2)	1 (1.3)/3 (3.9)
		Motzer *et al*[64]	2018	None	547	Renal cell carcinoma	145 (27)/ND	21 (4)/ND
Durvalumab	PD-L1	Antonia *et al*[65]	2017	None	473	NSCLC	87 (18.3)/ND	3 (0.6)/ND
		Motzer *et al*[66]	2018	None	475	NSCLC	88 (18.5)/ND	3 (0.6)/ND
		Loibl *et al*[67]	2019	None	92	Breast cancer	26 (28.3)/ND	3 (3.3)/ND
Atezolizumab	PD-L1	Herbst *et al*[68]	2014	None	277	Solid tumors or hematological malignancies	29 (10.5)/ND	0 (0)/ND
		Rosenberg *et al*[69]	2016	None	311	Urothelial carcinoma	24 (8)/3 (1)	1 (0.3)/2 (1)
		Fehrenbacher *et al*[70]	2016	None	142	NSCLC	ND	ND/2 (1)
		Socinski *et al*[71]	2018	ABCP	393	NSCLC	70 (17.8)	11 (2.8)
Avelumab	PD-L1	Chung *et al*[72]	2019	None	150	GC/GEJC	ND/2 (1.3)	ND/1 (0.7)[4]
		Barlesi *et al*[73]	2019	None	396	NSCLC	24 (6)/ND	0 (0)/ND

[1]Dose-limiting colitis was not observed in this trial;
[2]Progressed after ipilimumab;
[3]Immune-related event;
[4]No atezolizumab-related grade 4 but adverse events were reported, but only one patient showed Grade 5 cardiac failure. SCCHN: Squamous cell carcinoma of the head and neck; NSCLC: Non-small-cell lung cancer; ABCP: Atezolizumab plus bevacizumab plus carboplatin plus paclitaxel; GC/GEJC: Gastric/gastroesophageal cancer; ND: Not described.

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CASE REPORT

Successful management of adhesion related small bowel ischemia without intestinal resection: A case report and review of literature

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Abstract

BACKGROUND

Intraabdominal adhesions develop spontaneously or after an inflammatory process or surgical procedure in the abdomen. They are the most common cause of small bowel obstruction (SBO). SBO occasionally leads to intestinal ischemia (InIs) which can be a life-threatening condition that requires management as soon as possible. We herein report a case of SBO with InIs presented in our institution and treated without intestinal resection.

CASE SUMMARY

A 34-year-old man presented at the emergency department after a 12-h-onset diffuse abdominal pain, bloating and nausea. He had a history of traumatic right hepatectomy 11 years ago as well as adhesiolysis and resection of a long part of small bowel 2 years ago. An abdominal computed tomography (CT) showed dilated loops that led to the diagnosis of SBO. Due to deteriorating lactic acidosis, the patient was operated. Torsion of the small bowel around an adhesion led to 2.30 m of ischemic ileum. After the application of N/S 40 °C for 20 min, the intestine showed signs of improvement and it was decided to avoid resection and instead temporary close the abdomen with vacuum-pack technique. At the second-look laparotomy 48 h later, the intestine appeared normal. The patient was discharged on the 8th post-op day in excellent condition.

CONCLUSION

In case of SBO caused by adhesions, extreme caution is needed if InIs is present, as the clinical signs are mild and you should rely for diagnosis in CT findings and lactate levels. Conservative surgical approach could reverse the effects of InIs, if performed quickly, so that intestinal resection is avoided and should be used even when minimum signs of viability are present.

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Core tip: Intraabdominal adhesions are the most common cause of small bowel obstruction which occasionally leads to intestinal ischemia (InIs). InIs can be a life-threatening condition that requires high index of suspicion because the clinical signs are mild and you should rely for diagnosis in computed tomography findings and lactate levels. Lactate, if present is a good indicator of the progress or regression of the disease. Diagnosis of InIs leads to immediate operation. Conservative surgical approach avoiding resection of ischemic bowel, could lead to reversal of InIs and should be used even when minimum signs of viability are present.

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INTRODUCTION

Small bowel obstruction (SBO) is a major cause of hospital admissions (15% in United States) and is associated with significantly high rates of patients' morbidity and even mortality[1,2]. Post-operative abdominal adhesions play a pivotal role in SBO detection while 10% of affected patients are attributed to peritonitis and only 5% of SBO cases remain of unknown origin[2]. Generally, abdominal adhesions are created either spontaneously, by an inflammatory process or after a surgical procedure in the abdomen and pelvis[3,4]. Moreover, adhesive SBO (aSBO) occurs in 3% of all laparotomies and 1% during the first postoperative year. Recurrence rate after aSBO is 19%-53%[5]. Patients with SBO usually present with colicky abdominal pain, distention, nausea, vomiting, constipation and obstipation[1,6,7]. Laboratory findings include in the majority of the cases mild leukocytosis and possibly electrolyte disturbances[1,8]. Abdominal computed tomography (CT) remains the gold-standard diagnostic procedure for aSBO[9,10].

Initial management of aSBO is conservative including nasogastric tube for intestinal decompression with parallel administration of intravenous fluids and analgesics and electrolyte correction on occasion[5,11,12]. In accordance with recent investigations 70% of aSBO cases resolve without operative intervention[5]. However, one of the possible outcomes of SBO is intestinal strangulation which eventually leads to ischemia[5,13]. Intestinal ischemia (InIs) is a potentially lethal complication as it presents without specific symptoms and it requires high index of suspicion for establishment of the diagnosis[14]. Elevated lactate levels and acidosis are indicative of an upcoming ischemia and should also be taken into account during the patient's management[13,15-17]. Additional CT findings implying InIs are mesenteric edema, free peritoneal fluid and a transitional point, while parietal pneumatosis is a sign of irreversible bowel wall necrosis[2,13]. Thus, in case of evidence of small bowel strangulation, subsequent laparotomy is mandatory. We here present a case of aSBO with signs of intestinal strangulation and ischemia due to torsion around a dense adhesion at mid-ileum in a young male patient successfully treated without intestinal resection. Relevant literature is briefly reviewed. Clinical features and imaging findings are discussed, while the role of current diagnostic and therapeutic management of this nosologic entity is emphasized.

CASE PRESENTATION

Chief complaints

A 34-year-old man was admitted due to a 12-h-onset increasing diffuse abdominal pain.

History of present illness

The patient also appeared to our emergency department with general malaise and reported bloating and nausea.

History of past illness

He also reported similar clinical symptoms in the past. He was a 20 pack-year smoker. Moreover, the patient had a history of partial right hepatectomy due to liver trauma in 2007 and 1.5 m of small bowel resection due to aSBO in 2016.

Physical examination

On meticulous physical examination, he presented mild tachycardia, tachypnea as well as distended abdomen with guarding and diminished bowel sounds.

Laboratory testing

Apart from elevated lactate levels (Lac = 2.2 mmol/L), common laboratory blood and urine examinations were within normal limits.

Imaging examination

Subsequent ultrasonography was not indicative of pathologic findings. Erect abdominal X-rays depicted limited small bowel fluid levels, indicating intestinal obstruction. Subsequent CT scan identified the presence of bowel dilation and wall edema with parallel winding of the major vessels of gastrointestinal (GI) tract at the transitional zone, assessing InIs without signs of vascular obstruction or thrombosis.

FINAL DIAGNOSIS

Therefore, the final diagnosis was aSBO (Figure 1). Additional CT angiography as well as low molecular heparin administration was not implemented as signs of thrombosis were absent and intestinal strangulation was verified.

TREATMENT

Six hours after admission and fluid resuscitation, the patient was submitted to exploratory laparotomy, due to deteriorating lactic acidosis (Lac = 8 mmol/L) raising strong suspicion of evolving InIs. Surgical operation was performed via a midline incision while the presence of normal bowel from the ligament of Treitz to a length of 1.30 m of jejunum was verified. On the contrary, the rest of the small bowel including ileocolic junction was ischemic and congested due to twirling around an adhesion created at the location of an intestinal anastomosis. A diminutive sign of peristalsis was apparent at the most distal ischemic bowel loops (Figure 2). Resection was reserved as last treatment option due to the short length of the remaining healthy bowel. Rewarming with N/S (40 °C) was applied for 20 min. The intestine, slightly but clearly, improved in color and peristalsis in about half of its ischemic length. Lactic acidosis was also reduced (Lac = 6 mmol/L) during this operation allowing us to postpone resection. The abdomen was temporarily closed with vacuum-pack technique, searching for a second look laparotomy in forty-eight hours. During this period, lactate was normalized (Lac = 0.8 mmol/L).

OUTCOME AND FOLLOW-UP

Subsequent second look operation elucidated that the bowel was perfused in all its length and peristalsis was present (Figure 3). The abdomen was closed permanently. Hospital stay was uneventful and he was discharged from the surgical department 8 d postoperatively. Twenty-four hours later, the patient complained for bloody diarrheas, which was expected due to the mucosal apoptosis. Finally, the last 6 mo, the patient remains under intense monitoring without signs of relapse.

DISCUSSION

Intra-abdominal adhesions are fibrous bands between organs or tissues that are usually formed after an abdominal or pelvic operation, an inflammatory process, or an abdominal trauma[18]. In fact, it has been elucidated, that they may develop after abdominal surgeries in about 67% of the cases and in 97% of patients undergoing

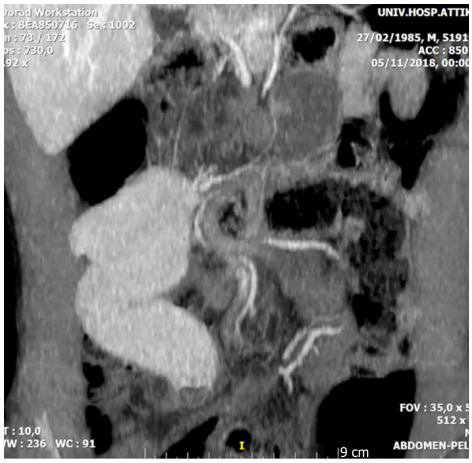

Figure 1 Abdominal computed tomography indicative of the torsion of the ileum around the adhesion at the center of the picture.

gynecological procedures[19,4]. However, the formation of adhesions varies among patients and depends on the severity and type of the initial surgery, potential post-operative complications as well as the presence of intra-peritoneal foreign bodies[3]. In general, according to a recent survey, the overall incidence of aSBO is 4.6%[20]. More specifically, aSBO complicates 19.3% among ileal-anal pouch anastomoses, while affects 11.1% of patients submitted to gynecological surgical operations. Nonetheless, the technique of the procedure affects significantly the development of SBO[20,21]. Consequently, aSBO prevalence was 9.5% in open colectomy versus 4.3% in laparoscopic one and 7.1% in open cholecystectomy *vs* 0.2% in relevant laparoscopic operation. Contrariwise, there was no difference in the development of aSBO after open or laparoscopic appendectomies (1.4% *vs* 1.3%)[20]. Finally, laparotomy due to abdominal trauma, was complicated by aSBO in 2.5% of the cases, whereas the incidence of early post-op SBO (within 30 days after surgery) was 24.2%[20,22]. Our case involves a 34-year-old man with a history of partial hepatectomy due to liver trauma 11 years ago, which was followed by a SBO and 1.5 m intestinal resection 2 years ago and now presented with aSBO and subsequent InIs.

Although the clinical presentation of aSBO depends on the location and duration of the obstruction, it usually involves colicky abdominal pain, abdominal distention, nausea and vomiting related to the site of intestinal strangulation[1,23]. Most patients report a history of prior abdominal surgery[24]. In case of ischemia though, intermittent abdominal pain becomes more severe and located while fever should always raise suspicion of InIs and sepsis[1,6]. Meticulous physical examination usually reveals a restless patient with signs of dehydration, poor skin turgor and abdominal distention[1,23]. During inspection visible peristalsis is sometimes observed. In subsequent auscultation, bowel sounds are initially increased and high-pitched, but diminish with advanced SBO[1]. Signs that SBO has resulted in InIs or perforation (peritonitis) include affected vital signs such as tachycardia, tachypnea and pyrexia as well as rebound tenderness, guarding and abdominal rigidity. Our patient presented with a 12-h-onset increasing diffuse abdominal pain, bloating and nausea and on examination mild tachycardia, tachypnea, distended abdomen with guarding and diminished bowel sounds were documented.

Laboratory findings suggestive of SBO more often include mild leukocytosis and possibly electrolyte disturbances due to dehydration and third space volume loss[8]. Elevated lactate levels and acidosis should be also considered through the patient's management as they strongly indicate intestinal strangulation[13]. In fact, lactate although not universally present at ischemia, when apparent is a good indicator of the progress or regression of the disease[25]. Imaging techniques that are proposed for aSBO vary according to the initial clinical signs, but mostly abdominal CT can contribute to the diagnosis of this condition as it may often accurately identify strangulation in aSBO[26,27]. Imaging findings may also include reduced bowel wall enhancement and mesenteric fluid detection indicative of strangulation as well as small bowel wall thickening associated with aSBO[28,29]. Ideally, only patients who develop strangulation should undergo surgery, and these operations should be performed promptly to avoid transmural necrosis and perforation. Early recognition of SBO strangulation is thus pivotal to help improve the patient outcome by preserving the involved bowel loops and avoiding needless surgical resection[30].

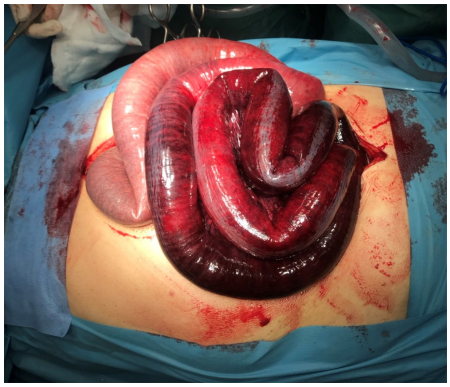

Figure 2 Dilated ischemic small bowel loops at the exploratory laparotomy.

Nevertheless, regardless of the cause, obstructed bowel eventually becomes edematous, leading to bowel ischemia, inflammation and necrosis. Strangulation is usually verified if at least one of the following criteria is fulfilled including bluish discoloration, loss of arterial pulsation, subserosal and mesenteric hemorrhage, lack of peristalsis and frank infarction. In our patient, abdominal CT scan revealed bowel dilation and wall edema with winding of the major vessels of GI tract predicting InIs.

Our case concerns a young man with aSBO and resultant ischemia of a 2.30 m long intestinal loop which involved the ileocecal valve. Thus, even though initial management of aSBO is usually conservative and the majority of aSBO cases resolve without surgical intervention, given the signs of InIs, laparotomy was mandatory[2,5,11]. The standard procedure for this condition involves resection of the ischemic bowel and primary anastomosis[31]. However, in the present case, resection would lead to short bowel syndrome; a mal-absorptive state caused by massive intestinal resection[32,33]. It usually occurs when the small bowel is less than 2 m long, so that its absorptive function is diminished and the patient cannot maintain its nutritional status and homeostasis by the enteral route alone. These patients are initially dependent on total parenteral nutrition which could progressively lead to liver insufficiency[32,34]. In order to avoid this outcome, taking into consideration that the physiologic consequences of ischemia are still reversible within six hours from onset, and based on diminutive sign of peristalsis at the ischemic ileum, it was decided initially a more conservative approach without intestinal resection[16]. The option of surgical excision was reserved for the second look laparotomy in case that the bowel became necrotic. Fortunately, at the second-look operation the small bowel appeared normal and the resection wasn't warranted. In this way and even though the intestinal loop at first seemed marginally viable judging from its color and size, the ischemia was reversed, and the patient improved quickly without complications.

CONCLUSION

The treatment of aSBO should be, at the beginning, conservative excluding cases presented with clinical signs or CT findings predictive of surgical intervention or peritonitis requiring an urgent laparoscopic or laparotomic exploration[2]. On occasion, conservative surgical approach avoiding resection of ischemic bowel, could lead to reversal of InIs and should be initiated even when minimum signs of viability are present and also when less than six hours have passed from the onset of ischemia, time limit allowing reversal of ischemic damage. The second look laparotomy in 24-48 h is an alternative that allows, under the condition of close monitoring (vital signs, clinical evaluation for acute abdomen, lactate levels), the reperfusion and resolution of ischemic impairment of a marginally viable bowel, or deterioration and early resection based on the close monitoring.

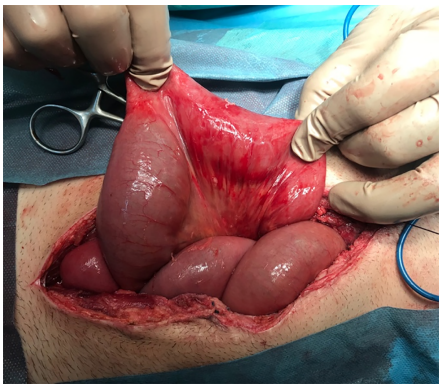

Figure 3 The small bowel at second look laparotomy 48 h later. Location of most severe ischemia: On the left side of the image the surgeon holds the bowel at the location of previous anastomosis were the adhesion that lead to torsion and ischemia was formed.

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ABOUT COVER

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EDITORIAL

Neutropenic enterocolitis: A clinico-pathological review

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Abstract

Neutropenic enterocolitis (NE) is a predominantly cecum-based disease with high mortality seen in patients post chemotherapy. The pathogenesis of NE is poorly understood and probably multifactorial involving mucosal injury, neutropenia, and impaired host defense to intestinal organisms. The clinical presentation is characterized as ileocolonic inflammation and bowel wall thickening in patients with neutropenia, fever, and abdominal pain. The pathological features of NE include patchy necrosis, hemorrhage, ulcer, edema, perforation, infiltrating organisms, and characteristically, depletion of inflammatory cells (neutrophils). NE should always be considered as a possible diagnosis in immunosuppressed patients, especially those receiving chemotherapy. High clinical and histological diagnostic discordance rate exists. High index of clinical suspicion and prompt appropriate personalized management are essential to achieve a lower mortality rate.

Key words: Neutropenic enterocolitis; Typhlitis; Chemotherapy; Neutropenia

Core tip: Neutropenic enterocolitis (NE) is a predominantly cecum-based disease with high mortality seen in patients post chemotherapy. The pathogenesis of NE is still poorly understood. The clinical presentation is characterized as ileocolonic inflammation and bowel wall thickening in patients with neutropenia, fever, and abdominal pain. The pathological features of NE include patchy necrosis, hemorrhage, ulcer, edema, perforation, infiltrating organisms, and characteristically, depletion of inflammatory cells (neutrophils). High clinical and histological diagnostic discordance rate exists. High index of clinical suspicion and timely diagnosis are critical for patient appropriate treatment and improvement of survival.

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INTRODUCTION

Neutropenic enterocolitis (NE) was also named as typhlitis, typhlenteritis, ileocecal syndrome, or cecitis. NE is a clinical entity described mostly in patients with hematologic malignancies, as well as other immunosuppressive causes such as acquired immune deficiency syndrome (AIDS), therapy for solid tumors, and organ transplant[1-6]. The clinical presentation is characterized as ileocolonic inflammation in patients with neutropenia, fever, and abdominal pain[5,6]. The cecum is the most commonly reported site to be involved[6,7]. The mortality of NE can reach up to 100% due to complications of malignancy, sepsis, or bowel necrosis and perforation[6,8]. In recently years, early recognition and progress in management have reduced mortality substantially[9,10]. A recent study showed a mortality rate of 32.1% in the intensive care units and a hospital mortality rate of 38.8%[11]. The clinical diagnosis of NE relies on major criteria and minor criteria. The major criteria include neutropenia, fever, and bowel wall thickening on computed tomography (CT) or ultrasound imaging. The minor criteria consist of nonspecific symptoms including abdominal pain, distension cramping, diarrhea, or lower gastrointestinal bleeding[11]. The NE patients are often in critical condition which may not permit endoscopic examination or surgical resection. Therefore, the pathologic features of NE have not been well recognized, and a high clinical and histological diagnostic discordance rate has been reported in NE cases[5].

CLINICAL CHARACTERISTICS AND IMAGING

The true occurrence rate of NE is unknown, which has been estimated at about 5% of patients hospitalized for leukemia/lymphoma, aplastic anemia or solid tumors treated with chemotherapy[12]. Neutropenia is the major risk factor for the development of NE. NE can develop in leukemia patients or patients in neutropenic condition receiving high dose chemotherapy to treat malignancies. Other conditions related to neutropenia include multiple myeloma, lymphoma, aplastic anemia, myelodysplastic syndromes, drug-induced neutropenia, cyclical neutropenia, agranulocytosis, and other immunosuppressive conditions such as AIDS and post-transplant patients[6,9]. NE patients usually present with nonspecific abdominal pain, diarrhea, nausea, vomiting, and abdominal distension[6,13]. Symptoms usually present 10-14 d post starting chemotherapy.

The initial diagnosis of NE is usually established by detection of the characteristic CT findings in neutropenic patients presenting with fever, abdominal pain and tenderness. The most common CT findings in NE is bowel wall thickening. Other CT findings include stranding mesentery, dilated bowel, enhancement of mucosa, and intestinal pneumatosis[6]. The abnormal findings mostly involve the ascending colon and cecum[14]. Plain films of the abdomen are nonspecific, but, are useful for detecting free air[6]. Occasionally, abdominal X-ray may show diffusely distended cecum and adjacent dilated small intestine, signs of thumbprinting, or localized pneumatosis intestinalis, suggestive of NE if clinical background fits[15]. Barium enema is contradicted in potential NE patients, due to its risk to cause bowel perforation[16,17]. In addition to CT scanning, detection of micro- organisms in blood and stool cultures and Clostridioides difficile toxin assays can help identify the superposed infection and decide the management of the patients[18].

PATHOLOGY FINDINGS

Histologic examination is the gold standard for the diagnosis of NE[19]. However, colonoscopy is relatively contraindicated in NE patients, as air insufflation may result in bowel perforation[5,20]. In patients who underwent colonoscopy examination or surgical resection of bowel segment, the gross findings in colonoscopy or surgical resection specimen include: Presence of patchy irregularity and nodularity, friable mucosa, and mass-like lesion that mimics malignancy[5,21]. Occasionally, NE causes

perforation of the bowel, a leading cause of death in NE[22]. The cecum and right colon are involved in nearly all the cases (Figure 1). Other bowel segments such as terminal ileum, transverse colon and left colon can also be variably involved. However, NE lesions are not reported in the appendix or rectum so far[5].

The key histopathologic features of NE are marked hemorrhagic necrosis, mucosal ulceration, extensive edema in the submucosa and laminal propria, marked congestion and even deep mural and transmural necrosis (Figure 2A-D)[5]. Most importantly, significant infiltrative inflammation should be absent in NE due to the profound neutropenia. Many cases are accompanied by infiltrating organisms like bacteria or fungus hyphae. However, inflammatory cell infiltration is still depleted despite in background of organism overgrowth[14]. Apoptotic bodies are not prominent in NE cases, distinguishing NE from the graft-versus-host disease, mycophenolate mofetil-induced injury and immune checkpoint inhibitor induced colitis[22].

PATHOGENESIS AND PATHOPHYSIOLOGY

The pathogenesis of NE is poorly understood and probably multifactorial involving mucosal injury, neutropenia, and impaired host defense to intestinal organisms. The initial morbidities of the patients lead to intestinal edema, engorged vessels, and mucosal surface disruption, especially of the ileocolonic segments. Chemotherapeutic agents like cytarabine can directly cause mucositis or predispose to intestinal distension and necrosis, and consequently impair the intestinal motility. The initial intestinal mucosal injury in the background of immunocompromised state of the afflicted patients leads to intestinal edema, vascular dilation, mucosal disruption, and bacterial intramural invasion[5-7]. Moreover, intestinal leukemic infiltration may be a superposed factor in the pathogenesis of NE[23]. Neutropenia and the use of steroids complicate the situation by reducing host defenses against infection. Microbes including variety of bacteria, fungi and cytomegalovirus have been implicated as causes[24-26].

DIFFERENTIAL DIAGNOSIS

Clinically and radiologically, many conditions can overlap with NE and its mimics including acute appendicitis, appendiceal abscess, ischemic enteritis, *Clostridium difficile* colitis, graft-versus-host disease, mycophenolate mofetil-induced injury, and enteric involvement by lymphoma/leukemia[5,6]. Recent high-dose chemotherapy along with the symptoms of fever and abdominal pain, lab finding of neutropenia, and imaging finding of bowel wall thickening will aid the diagnosis of NE. A timely diagnosis and proper management will significantly improve the prognosis[11]. Histopathological findings are the gold standard to distinguish NE from its mimics, if diagnostic tissue especially resection specimen is available for pathology examination. In a multi-institutional histological study of NE lesions, high discordance rate (35%) between clinical and pathologic diagnoses has been reported. Fifteen percent of patients with histologically confirmed NE were not clinically suspected for NE, whereas, twenty six percent of patients with clinically suspected NE were histologically evaluated as non-NE[5].

TREATMENT CONSIDERATIONS

So far, there is no high-quality prospective or retrospective studies on the treatment of NE, and therefore, no recommended uniform management strategy or guidelines for NE yet. A general approach to patients with NE should be individualized depending on the complications and pre-morbidities of the patients. Treatment of NE patients without significant complications such as peritonitis, perforation, or bleeding is mainly supportive including bowel rest and intravenous fluids supplement, and use of antibiotics[11]. Neutropenia is the major risk factor for the pathogenesis of NE. Cytopenia, coagulopathy, and mucosal hemorrhage can cause coagulopathy. Therefore, coagulation should be monitored in patients with NE and be corrected promptly[7,11]. The use of granulocyte colony stimulating factor can hasten neutrophil recovery and may be beneficial in some patients[7,11]. Surgical intervention has been recommended for patients with evidence of bowel perforation, persistent bleeding even after correction of cytopenia and coagulopathy abnormalities, clinical deterioration in spite of the intensive medical intervention, and the presence of other

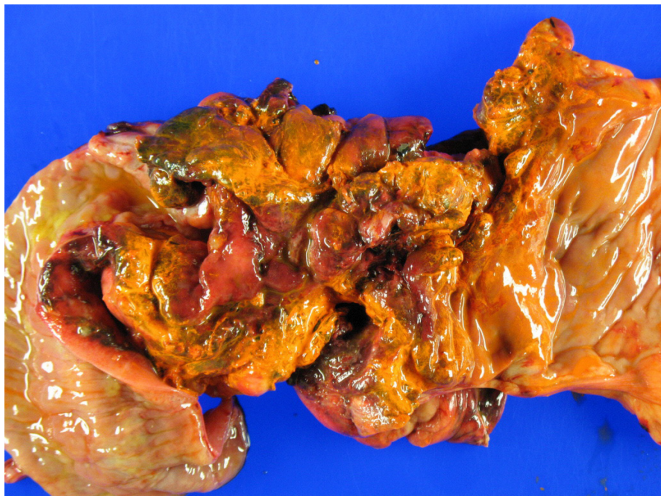

Figure 1 Gross findings of neutropenic enterocolitis. Cecal mucosa with patchy hemorrhage, necrosis and ulceration.

surgical conditions, *e.g.*, abscess and acute appendicitis[25,27]. Interestingly, patients who recovered from NE are at risk from developing NE again during subsequent chemotherapy. Patients should be fully recovered from NE before a new chemotherapy starts[25].

CONCLUSION

Neutropenic enterocolitis is a predominantly cecum-based disease with high mortality seen in patients post chemotherapy. The diagnosis of NE should always be considered in immunosuppressed patients, particularly those treated with chemotherapy when they present with appropriate symptoms. The pathogenesis of NE is still unclear and probably multifactorial involving mucosal injury, neutropenia, and impaired host defense to intestinal organisms. The clinical presentation is characterized as colonic inflammation and bowel wall thickening in patients with neutropenia, fever, and abdominal pain. The pathological features of NE include patchy necrosis, hemorrhage, ulcer, edema, perforation, infiltrating organisms, and characteristically, depletion of neutrophil infiltration. High clinical and histological diagnostic discordance rate exists. The differential diagnoses of NE include non-specific chronic and acute colitis, graft-versus-host disease associated colitis, malignancy relapse, drug-induced colitis, acute appendicitis, and bowel ischemia. Conservative treatment or surgical intervention should be tailored to the individual patient. High index of clinical suspicion and prompt appropriate treatment is essential to achieve a lower mortality rate.

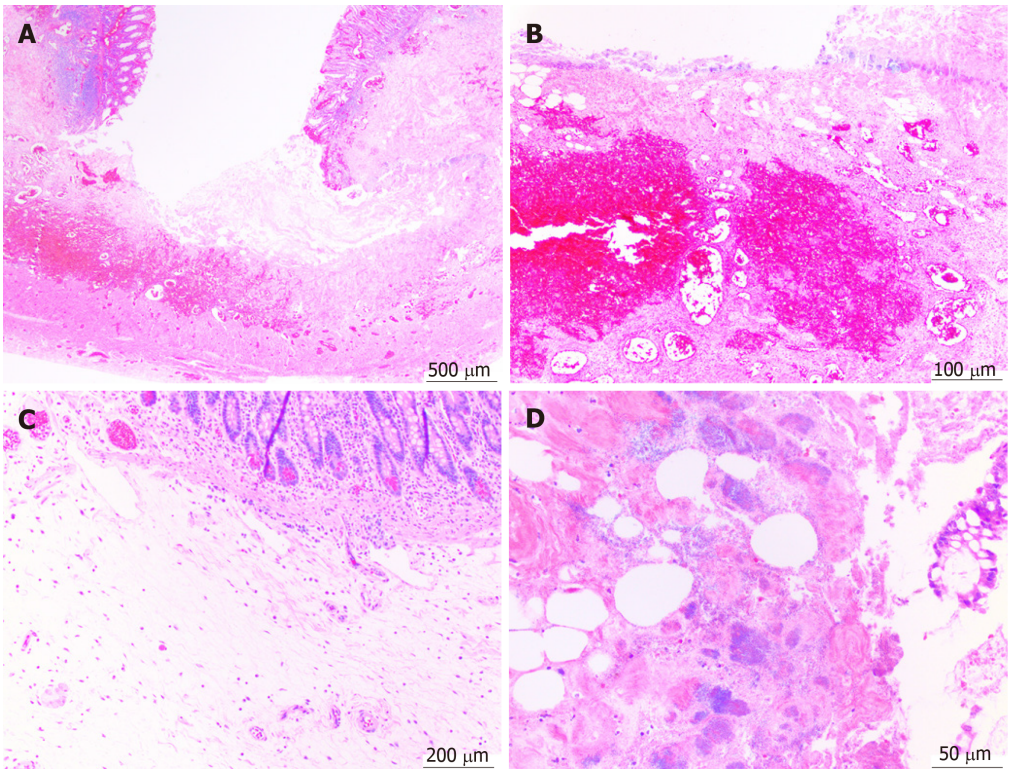

Figure 2 Histologic features of neutropenic enterocolitis. A: Ulceration; B: Hemorrhage and congestion; C: Prominent submucosal edema with paucity of infiltrative inflammatory cells; D: Infiltrative bacteria. Hematoxylin-eosin stain.

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ORIGINAL ARTICLE

Observational Study

Comparison of cytokine and phosphoprotein profiles in idiopathic and Crohn's disease-related perianal fistula

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Author contributions: Haddow J MacDonald TT and Knowles CH conceived and designed the study; Haddow J recruited the patients and acquired the data; Haddow J and Knowles CH acquired the specimens; Haddow J and Musbahi O performed the laboratory measurements and inputted the data; Haddow J and Knowles CH analysed the data; Haddow J, Knowles CH and MacDonald TT interpreted the data; Haddow J wrote the article; Haddow J, Musbahi O, MacDonald TT and Knowles CH edited, reviewed and approved the final article.

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Abstract

BACKGROUND

Perianal fistulae are either primary (idiopathic) or secondary [commonly associated with Crohn's disease, (CD)]. It is assumed, although not proven, that the pathophysiology differs.

AIM

To systematically compare the clinical phenotypes, cytokine and phosphoprotein profiles of idiopathic and CD-related perianal fistulae.

METHODS

Sixty-one patients undergoing surgery for perianal fistula were prospectively recruited (48 idiopathic, 13 CD) into a cohort study. Clinical data, including the Perineal Disease Activity Index (PDAI) and EQ-5D-5L were collected. Biopsies of the fistula tract, granulation tissue, internal opening mucosa and rectal mucosa were obtained at surgery. Concentrations of 30 cytokines and 39 phosphoproteins were measured in each biopsy using a magnetic bead multiplexing instrument and a chemiluminescent antibody array respectively. Over 12000 clinical and 23500 laboratory measurements were made.

RESULTS

The PDAI was significantly higher (indicating more active disease) in the CD group with a mean difference of 2.40 (95%CI: 0.52-4.28, $P = 0.01$). Complex pathoanatomy was more prevalent in the CD group, namely more multiple fistulae, supralevator extensions, collections and rectal thickening. The IL-12p70 concentration at the internal opening specimen site was significantly higher (median difference 19.7 pg/mL, 99%CI: 0.2-40.4, $P = 0.008$) and the IL-1RA/IL-1β ratio was significantly lower in the CD group at the internal opening specimen site (median difference 15.0, 99%CI = 0.4-50.5, $P = 0.008$). However in the

the corresponding author.

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remaining 27 cytokines and all 39 of the phosphoproteins across the four biopsy sites, no significant differences were found between the groups.

CONCLUSION
CD-related perianal fistulae are more clinically severe and anatomically complex than idiopathic perianal fistulae. However, overall there are no major differences in cytokine and phosphoprotein profiles.

Key words: Anal fistula; Crohn's disease; Cytokines; Phosphoproteins; Pathogenesis

Core tip: We systematically compared idiopathic and Crohn's perianal fistulae, but did not find major differences in their cytokine and phosphoprotein profiles. Although more research is needed, our results support the thesis that biological agents effective in Crohn's disease-related perianal fistulae may also have a role in selected surgically-intractable idiopathic perianal fistulae.

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INTRODUCTION

Perianal fistulae occur in 1.04 to 2.32 per 10000 people per annum[1,2], are more common in males (ratio about 2:1), and cause significant physical and psychosocial morbidity. Fistulotomy can achieve successful healing at one year in 75% of patients[3], but is not always possible when the fistula involves a significant portion of the anal sphincter complex. Sphincter-sparing operations such as a fistula plug, advancement flap and ligation of the internal fistula tract only have success rates at one year of 55%, 53% and 42% respectively[3]. In Crohn's disease (CD), perianal fistulae can heal in response to biological agents in 68% of patients[4]. Treatments for perianal fistulae could be improved if we had a better understanding of their pathophysiology.

The aetiology and pathophysiology of perianal fistulae is still unclear. The majority (90%) are considered to be primary or idiopathic[5]. In such patients, Parks' cryptoglandular theory prevails, supposing infection of an anal gland as the primary lesion. Suppuration then penetrates through the internal anal sphincter creating a fistula tract[6].

In contrast, secondary fistulae, which can be associated with inflammatory bowel disease, tuberculosis and human immunodeficiency virus[5,7]. are assumed to arise from inflammation of the anorectal mucosa. By far the most common association is with CD, where perianal involvement is found in around one third of patients[8]. Scharl and Rogler summarised the immunological evidence for the pathogenesis of fistulae in CD[9]. In their proposed mechanism, an epithelial defect caused by inflammation or injury allows pathogen-associated molecular patterns from the microbiota to gain entry to the lamina propria and induce various pathways mediated by TNF-α, transforming growth factor beta (TGF-β), IL-13, matrix metalloproteinases (MMPs) and integrin-αvβ6. These drive epithelial-to-mesenchymal transition, which allows cell invasion and migration, resulting in a penetrating fistula tract lined by transitional cells.

Research to date into the pathophysiology of idiopathic and CD-related perianal fistula has evolved separately due to the underlying assumption that they are fundamentally different. Some previous studies made the comparison between these two types by use of specific histological, microbiological and immunological methods[10-13]. Overall their conclusions supported this underlying assumption, but testing this assumption was not their objective. We therefore hypothesised that idiopathic and CD-related perianal fistulae are different and aimed to test this systematically by comparing their clinical phenotypes, cytokine and phosphoprotein profiles.

MATERIALS AND METHODS

Patients

We conducted a prospective cohort study between March 2014 and April 2015 within a NHS University Hospital (approved by Queen's Square Research Ethics Committee, reference number 14/LO/0071). Adults with an idiopathic or CD-related perianal fistula requiring surgical intervention were included. Rectal, intestinal and subcutaneous fistulae were excluded.

One hundred and thirty consecutive patients were identified from outpatient and inpatient referrals, multidisciplinary meetings and surgical waiting lists. Twenty-two were not approached (simply for logistic reasons), 28 were ineligible and 11 declined. Thus we recruited 61 patients, 48 (79%) idiopathic and 13 (11%) CD-related (Supplementary Figure 1).

Patient characteristics were in keeping with the general epidemiology (Supplementary Table 1). None had a stoma. In the CD group, six had previous abdominal fistulae, six had previous abdominal surgery, and seven were naïve to anti-TNF-α antibody therapy. All had a combination of radiological, endoscopic and histological features of CD. Idiopathic patients had CD excluded by radiological and endoscopic imaging in 32 (67%) and clinical assessment alone in 16 (33%). Reasons for not imaging patients were patient declined (4) and not clinically indicated (16).

Healthy controls

For the purposes of studying the rectal biopsies, we approached nine consecutive patients undergoing diagnostic colonoscopy for non-inflammatory conditions. Two declined and one was excluded due to ileal inflammation found during colonoscopy. Thus we recruited six healthy controls for rectal biopsies (Supplementary Table 2). Five were female and the mean age was 53.5 years (SD = 20.6).

Data and specimen collection

We collected clinical information, Perineal Disease Activity Index (PDAI)[14], EuroQol EQ-5D-5L[15], and intra-operative findings, which were assisted by pre-operative magnetic resonance imaging in 49 (80%) patients. PDAI and EQ-5D-5L at baseline were measured in all but one participant.

Biopsy specimens were taken from: (1) The fistula tract wall; (2) Tract granulation tissue (if present); (3) Internal opening mucosa (if present); and (4) Rectal mucosa. Specimens were kept on ice and processed within four hours. One patient did not undergo biopsy due to unavailability of research staff.

Processing of tissue specimens

Fresh specimens were prepared under a microscope. Those reserved for phosphoprotein quantification were snap frozen and stored at -80 °C. Those reserved for cytokine quantification were immediately incubated in 300 μL serum free HL-1 medium (Lonza, Cambridge, United Kingdom) containing 10 U/mL penicillin and streptokinase, 32 μg/mL gentamicin and 1 in 100 Lglutamine (Sigma-Aldrich, Gillingham, United Kingdom) at 37 °C and 5% carbon dioxide for 24 h. The supernatant was stored at -80 °C.

Cytokine quantification

We used 30-plex Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, United States) to quantify the concentrations of 30 cytokines and chemokines: EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17, IL-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, RANTES, and VEGF (Supplementary Table 3). Measurements were made using a MAGPIX multiplexing instrument (Luminex Corporation, Austin, TX, United States). We followed the manufacturer's recommended quality control procedures to ensure validity[16]. Specimen supernatants were analysed in singlet due to resource constraints, however, quality controls were measured in duplicate and 95% of these were within the accepted margin of error.

Phosphoprotein quantification

The method of phosphoprotein quantification was based on previous work within our laboratory[17]. Cells were lysed using 100 μL of radioimmunoprecipitation assay buffer containing one microlitre of Phosphatase Inhibitor Cocktail 2 and one microlitre of protease inhibitor in dimethyl sulfoxide (Sigma-Aldrich, Gillingham, United Kingdom) and a manual ultrasonic cell disruptor intermittently for 10 min at 4 °C. We then separated off the supernatant and performed a Bradford Assay (Bio-Rad Laboratories Inc., Hercules, CA, United States) to estimate the protein concentration.

PathScan RTK Signaling Antibody Array, Chemiluminescent Readout (Cell Signaling Technology, Danvers, Massachusetts) was used to quantify the phosphorylation status of 28 RTKs and 11 signalling nodes: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, FGFR1, FGFR3, FGFR4, InsR, IGF-IR, TrkA/NTRK1, TrkB/NTRK2, Met/HGFR, Ron/MST1R, Ret, ALK, PDGFR, c-Kit/SCFR, FLT3/Flk2, M-CSFR/CSF-1R, EphA1, EphA2, EphA3, EphB1, EphB3, EphB4, Tyro3/Dtk, Axl, Tie2/TEK, VEGFR2/KDR, Akt/PKB/Rac (at Thr308), Akt/PKB/Rac (at Ser473), p44/42 MAPK, S6 Ribosomal Protein, c-Abl, IRS-1, Zap-70, Src, Lck, Stat1 and Stat3 (Supplementary Table 4). A total of 113 μg of protein from each whole cell lysate was probed on the array. The chemiluminescent signals were detected on Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom), digitised using a lightbox and camera (Nikon D70 digital camera with a Nikon 18-70 mm 1:3.5-4.5G DX lens, Nikon Corporation, Tokyo, Japan) and measured using ImageQuant TL 2005 (GE Healthcare Life Sciences, Pittsburg, PA, United States). The measurements were normalised to give pixel intensities ranging from zero to 100%. Prior experiments determined a positivity threshold of 20%.

Statistical analysis

Sample size was based on feasibility (recruitment period). Further, there is little consensus on sample size estimations for assays used in this study and no prior relevant data upon which to base a calculation.

Data were entered into a validated database using Access 2010 (Microsoft, Redmond WA, United States). Double-entering of a random 10% data sample estimated the error rate at 0.6%. Proprietary software (SPSS Statistics version 22, IBM, Armonk, NY, United States) was used to analyse data, and Excel 2010 (Microsoft, Redmond, WA, United States) to produce the heat map charts. Parametric and non-parametric methods were used for normal and non-normal data respectively. We considered $P < 0.05$ to be statistically significant. When comparing the cytokine and phosphoprotein data where multiple comparisons were made, using $P < 0.01$ as statistically significant was considered an appropriate correction. This study was reviewed by a biomedical statistician. The STROBE guidelines were followed for reporting[18].

RESULTS

Clinical data

The PDAI was significantly higher (indicating more active disease) in the CD group with a mean difference of 2.40 (95%CI: 0.52-4.28, $P = 0.01$). The EQ visual analogue scores (VAS) and index values were similar between the groups. Multiple fistulae were more prevalent in CD patients (23% *vs* 4%). The distribution of the different types of fistulae under the Parks' classification was the same between the groups, with trans-sphincteric being by far the commonest. Prevalence of a high primary tract and horseshoe extensions were similar between groups. However, supralevator extensions and collections were commoner in the CD group (31% *vs* 6% and 92% *vs* 33% respectively). Rectal thickening, reflecting proctitis, was almost exclusively observed in the CD group (Table 1).

Cytokine profiles

All four specimen sites yielded substantial levels of IL-1RA, IL-6, MCP-1, RANTES, VEGF, G-CSF and IL-8. GM-CSF, IFN-α2, IP-10, MIP-1α and MIP-1β were also moderately abundant in the granulation tissue, internal opening and rectal mucosa. Granulation tissue, compared to the other specimen sites, yielded higher concentrations of G-CSF, IL-10, IL-1RA, and IL-1β (Figure 1).

Only two cytokines demonstrated significant differences between the idiopathic and CD groups. IL-12p70 concentration at the internal opening was higher in patients with CD (28.3 pg/mL, IQR = 7.4-50.1 *vs* idiopathic 7.4 pg/mL, 4.6-12.7). The median difference was 19.7 pg/mL (99%CI: 0.2-40.4, $P = 0.008$). The IL-1RA/IL-1β ratio was significantly lower in CD group at the internal opening (3.3, IQR = 1.8-7.6 *vs* idiopathic 19.0, 4.3-51.2). The median difference was 15.0 (99%CI: 0.4-50.5, $P = 0.008$) (Figure 2).

There were no significant differences between the groups for any other cytokine concentrations at the four specimen sites. There were also no significant differences in the cytokine concentrations in the rectal mucosa between the CD group and the healthy controls (Supplementary Figure 2).

Phosphoprotein profiles

All four specimen sites yielded signals across both patient groups for EGFR, HER2,

Table 1 Clinical features

	Idiopathic	Crohn's Disease	*P* value
Mean PDAI (SD)	5.67 (2.76)	8.07 (3.99)	0.01[a]
Mean EQ VAS (SD)	71.2 (21.1)	63.4 (20.2)	0.22
Mean EQ index (SD)	0.734 (0.259)	0.715 (0.214)	0.80
Seton *in situ*, *n* (%)	21 (44)	3 (23)	0.22
Number of fistulae, *n* (%)			0.09
Single unbranched	31 (65)	3 (23)	
Single branched	15 (31)	7 (54)	
Multiple	2 (4)	3 (23)	
Parks' classification, *n* (%)			0.54
Inter-sphincteric	7 (15)	1 (8)	
Trans-sphincteric	38 (79)	10 (77)	
Supra-sphincteric	3 (6)	2 (15)	
High primary tract	15 (31)	6 (46)	0.34
Secondary tract(s), *n* (%)			0.02[a]
None	31 (65)	4 (31)	
Infralevator	14 (29)	5 (38)	
Supralevator	3 (6)	4 (31)	
Horseshoe, *n* (%) extension(s)	12 (25)	5 (38)	0.49
Collection(s) , *n* (%)	16 (33)	12 (92)	< 0.001[a]
Thickened rectum, *n* (%)	1 (2)	7 (54)	< 0.001[a]

Perineal Disease Activity Index, EuroQoL visual analogue score and index measured at pre-operative clinic assessment. Continuous data was compared using *t*-test. Discrete data compared using Fisher's Exact test. $P < 0.05$ was considered significant.

[a]$P < 0.05$. PDAI: Perineal Disease Activity Index; SD: Standard deviation; EQ: EuroQol; VAS: Visual analogue score.

HER3, FGFR1, FGFR3, FGFR4, Stat1, and Stat3. In the rectal mucosa, we observed positive signals for InsR, IGF-1R, c-Kit, Tie2, Akt at phosphorylation site Thr308, S6 Ribosomal Protein, IRS-1 and Src in both patient groups and the healthy controls. There were no significant differences in phosphoprotein levels between the patient groups at any of specimen sites (Figure 3).

When comparing the CD group with the healthy controls at the rectal mucosa specimen site, the levels of six phosphoproteins were significantly higher in the healthy controls: EphA1, EphA2, EphB1, EphB4, Tyro3 and VEGFR2 (Supplementary Figure 3). Example microarray images are shown in Figure 4. There were no differences the remaining 33 phosphoproteins.

DISCUSSION

To our knowledge, this is the largest study to date to systematically compare idiopathic and CD-related perianal fistulae in a well-defined patient cohort. Both groups displayed a broad distribution of disease characteristics from simple to complex.

CD-related perianal fistulae were clinically more severe

In the CD group, the PDAI was significantly higher and complex pathoanatomy was more prevalent, supporting the commonly-held belief that CD-related perianal fistulae are more severe and complex than idiopathic. However, this did not translate to differences in EQ-5D, probably because this generic health measure lacked sensitivity to demonstrate a relatively small clinical difference.

Cytokine and phosphoprotein profiles of idiopathic and CD-related perianal fistulae were similar

Detailed profiling of 30 different cytokines and chemokines and 39 different phosphoproteins at four different biopsy sites showed that the idiopathic and CD groups were broadly similar. Our cytokine profiling is comparable to and substantially more extensive than previous studies[13,19,20] and our phosphoprotein

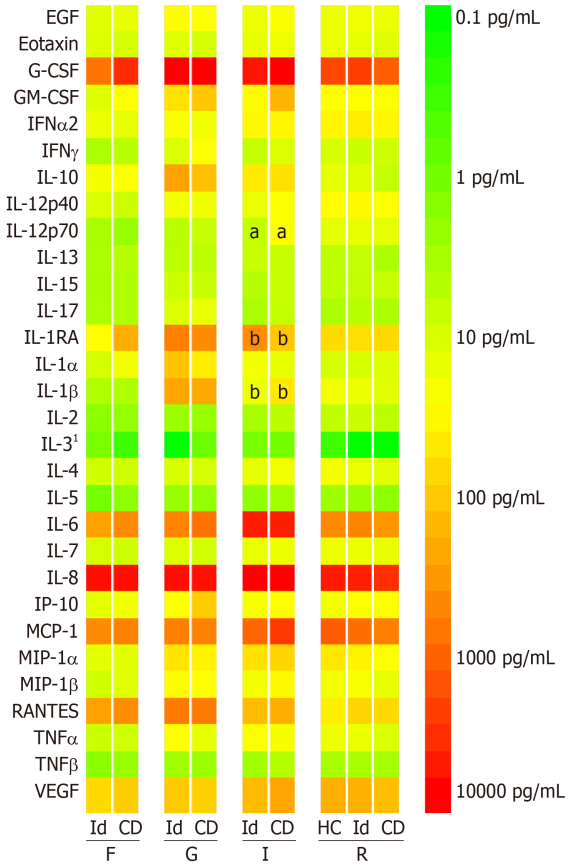

Figure 1 Median cytokine concentrations (log-10 scale) in the supernatants from 24-h fresh tissue cultures using a 30-plex Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, United States) and a MAGPIX multiplexing instrument (Luminex Corporation, Austin, TX, United States). The median IL-12p70 concentration at the internal opening was higher in the Crohn's disease group (a). The median difference was 19.7 pg/mL (Hodges-Lehman, 99%CI: 0.2-40.4; Mann-Whitney *U*, *P* = 0.008). The IL-1RA/IL-1β concentration ratio was significantly lower in the Crohn's disease group at the internal opening (b). The median difference was 15.0 (99%CI: 0.4-50.5, *P* = 0.008). There were no other statistically significant differences between the groups in the remaining 27 cytokines at the four biopsy sites. [1]Results should be interpreted cautiously as they were below the minimum detectable concentration for the assay. Id: Idiopathic; CD: Crohn's disease; HC: Healthy controls; F: Fistula tract; G: granulation tissue; I: Internal opening; R: Rectal mucosa.

profiling is the first to be reported in perianal fistula disease.

Only four previous studies directly compared idiopathic and CD-related perianal fistulae. Bataille *et al*[10,11] found both types had a lining of granulation tissue containing histiocytes and capillaries, a lumen filled with nuclear debris, neutrophils and lymphocytes, and markers indicative of epithelial-to-mesenchymal transition. However, CD-related perianal fistulae featured more CD45R0 positive T cells and CD20 positive B cells, whilst idiopathic fistulae featured more CD68 positive macrophages. Kirkegaard *et al*[12] found both types had similar MMP-3 and MMP-9 upregulation. Tozer found both types had similar levels of IL-2, IL-4, IL-6, IP-10, TNF-α and IFN-γ in tissue culture supernatants, but also reported higher numbers of T cells, lower expression of dendritic cell homing markers and fewer CD65 positive macrophages in CD-related perianal fistulae[13].

It is well established that there are significant differences in the cytokine expression in CD-affected intestinal mucosa, compared with healthy mucosa, namely pro-inflammatory cytokines IL-1, TNF-α, IL-6, IL-8, IL-12, IL-17 and IL-21, and anti-inflammatory cytokines IL-10 and TGF-β[21]. Thus, notwithstanding Tozer's results mentioned above, similar differences might be seen in idiopathic compared with CD-related perianal fistulae. To broaden this line of enquiry, we analysed the tissue culture supernatants for 30 different cytokines. Only two measurements showed a significant difference between the groups: IL-12p70 and the IL-1RA/IL-1β ratio, both at the internal opening.

IL-12 is a proinflammatory cytokine produced by dendritic cells and macrophages. It comprises two subunits, IL-12p35 and IL-12p40, which combine into IL-12p70. The IL-12 receptor is found mainly on T cells and natural killer cells. IL-12 induces

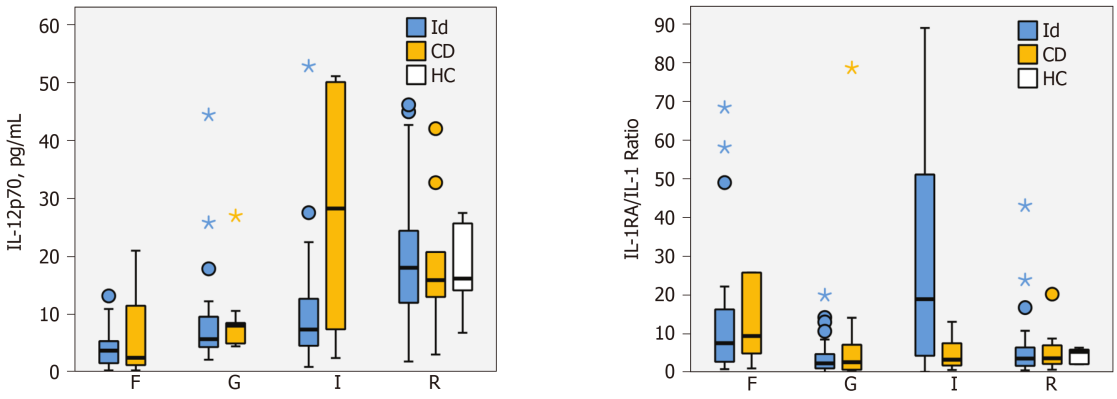

Figure 2 Boxplots for IL-12p70 concentration (left) and IL-1RA/IL-1β concentration ratios (right) in the supernatants from 24-h fresh tissue cultures using a 30-plex Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, United States) and a MAGPIX multiplexing instrument (Luminex Corporation, Austin, TX, United States). At the internal opening, differences between the idiopathic and Crohn's disease groups were statistically significant. Median difference in IL-12p70 concentration at the internal opening was 19.7 pg/mL (Hodges-Lehman, 99%CI: 0.2-40.4; Mann-Whitney *U*, *P* = 0.008). The median difference in IL-1RA/IL-1β at the internal opening was 15.0 (Hodges-Lehman, 99%CI: 0.4-50.5; Mann-Whitney *U*, *P* = 0.008). Circle marker, outlier within 1.5 × interquartile range (IQR); Star marker, outlier out with 1.5 × IQR. One outlier not shown to limit Y-axis scale.

production of IFN-γ, promotes T helper 1 cell differentiation and forms a link between innate resistance and acquired immunity[22]. It plays an important role in CD pathogenesis. The expression of IL-12 is upregulated in CD mucosa[23]. This study found significantly higher concentrations of IL-12p70 at the internal opening in the CD group, suggesting a similarly important role for IL-12 in CD-related perianal fistula and may represent a difference in its pathophysiology compared with idiopathic perianal fistula disease. However, these data need to be interpreted with caution, as the 99% confidence interval came close to zero.

IL-1α and IL-1β mediate immune and inflammatory responses. They are primarily produced by tissue macrophages, monocytes, fibroblasts, and dendritic cells and enable transmigration of immune cells to the site of inflammation. IL-1β concentration is increased in inflamed CD colonic mucosa[24]. We found low concentrations in the fistula tract, internal opening and rectal mucosa, with no significant differences between the groups. This might suggest that the role of IL-1 in perianal fistula differs from inflamed CD colonic mucosa. However, we found the IL-1RA/IL-1ß ratio at the internal opening was significantly lower in the CD group, which is in keeping with previous reports in inflamed CD colonic mucosa[24].

RTKs and intracellular signalling pathways control and regulate cell behaviour[25]. Many intracellular signalling pathways are upregulated in CD[17]. For example, signal transducer and activator of transcription 4 in T helper 1 cells is activated by IL-12, driving IFN-γ and TNF-α production[26]. Indeed an anti-IL-12 monoclonal antibody, ustekinumab, is of clinical benefit in CD[27]. We analysed 39 different phosphoproteins involved in epithelial cell signalling, wound healing, inflammation and T cell activation, and found no significant differences in the phosphoprotein profiles between idiopathic and CD-related perianal fistulae. We were also interested to see if fistula immunopathology changes over time. However, due to only ten patients having fistulae less than 12 mo old, this subgroup analysis was not possible.

Cell signalling in rectal mucosa may be suppressed in both idiopathic and CD-related perianal fistula disease

Comparison of the rectal mucosa in the idiopathic and CD groups with healthy controls found no differences in the cytokine profiles. However, in the rectal mucosa, the signals in both disease groups appeared to be suppressed compared with the healthy controls. This difference was significant for six phosphoproteins (EphA1, EphA2, EphB1, EphB4, Tyro3 and VEGFR2) when comparing the healthy controls with the CD group.

The first four are RTKs from the Ephrin subfamily. Ephrin signalling is important in controlling cellular proliferation in the crypts and differentiation of enterocytes on the villi. Loss of ephrin receptor and ephrin signalling appears to contribute to wound healing defects as in inflammatory bowel disease[28]. Our study suggests that suppression of ephrin receptor expression in the rectal mucosa may be a pathological feature in patients with both idiopathic and CD-related perianal fistulae. The consequence of this conclusion is potentially important for two reasons: (1) Idiopathic and CD-related perianal fistulae may be similar in their immunopathology with regards to ephrin signaling; and (2) Abnormalities in those with idiopathic perianal

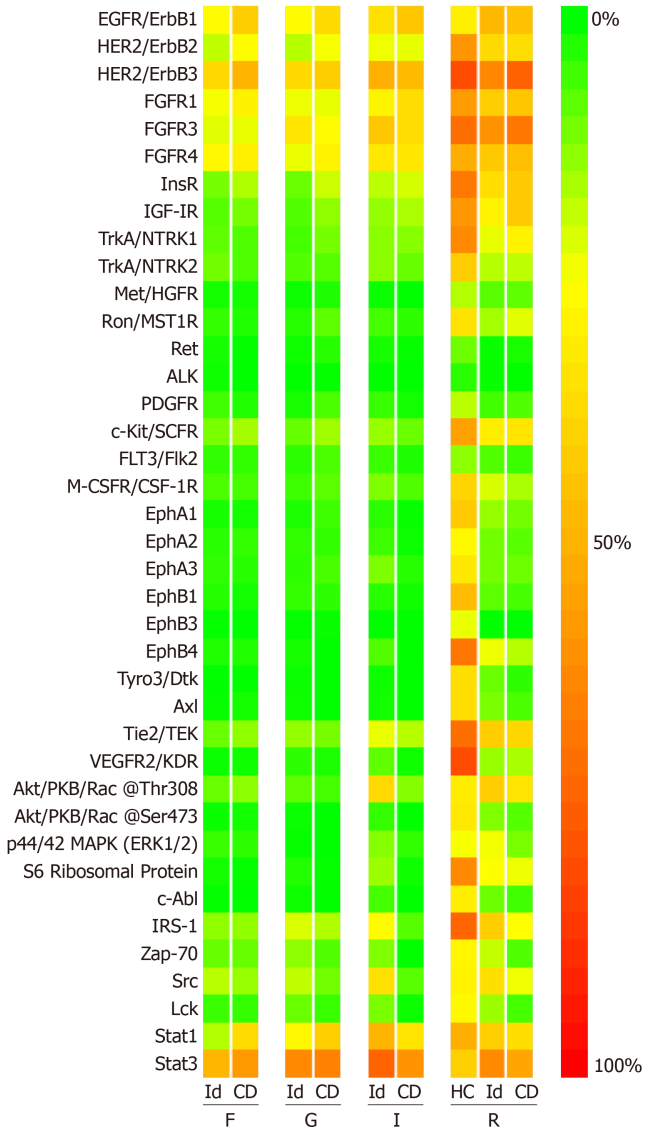

Figure 3 Median signal intensities (%) quantifying the phosphorylation status of receptor tyrosine kinases in the cell lysates of the fresh tissue samples using the PathScan RTK Signaling Antibody Array, Chemiluminescent Readout (Cell Signaling Technology, Danvers, Massachusetts). The signal intensities were similar in the idiopathic and Crohn's disease groups at the four specimen sites. When the Crohn's disease group was compared to healthy controls, a statistically significant difference (Mann Witney *U*, $P < 0.01$) was seen for EphA1, EphB1, EphB4, Tyro3 and VEGFR2. Id: Idiopathic; CD: Crohn's disease; HC: Healthy controls; F: Fistula tract; G: Granulation tissue; I: Internal opening; R: Rectal mucosa.

fistulae might not be confined to the peri-fistula tissue, a notion that has not previously been reported.

Tyro3 is a RTK that, along with Axl and Mertk, and their ligands Gas6 and Protein S, makes up the Tyro3-Axl-Mertk (TAM) signalling pathway. This pathway is involved in the negative regulation of inflammation, removal of apoptotic cells and potential induction of the tissue repair response. In inflammatory bowel disease, TAM signalling is suppressed, which, in the presence of mucosal injury, leads to an accumulation of apoptotic neutrophils and a failure of macrophages to acquire an alternative activation state[29]. Our study with respect to the rectal mucosa in the CD group is consistent with this. However, it also suggests that suppression of Tyro3 may also occur in the rectal mucosa of those with idiopathic perianal fistulae, further supporting the notion described above.

VEGFR2 is the principle RTK that transmits VEGF signals in the vascular endothelium. The principle effect of VEGFR2 activation is angiogenesis, which is a feature of both health and disease. VEGFR2 may be suppressed in quiescent CD colonic mucosa, compared with healthy controls[30]. In the rectal mucosa, we found that VEGFR2 levels were lower in patients both with idiopathic and CD-related perianal fistulae, compared with healthy controls. This suggests that expression of this

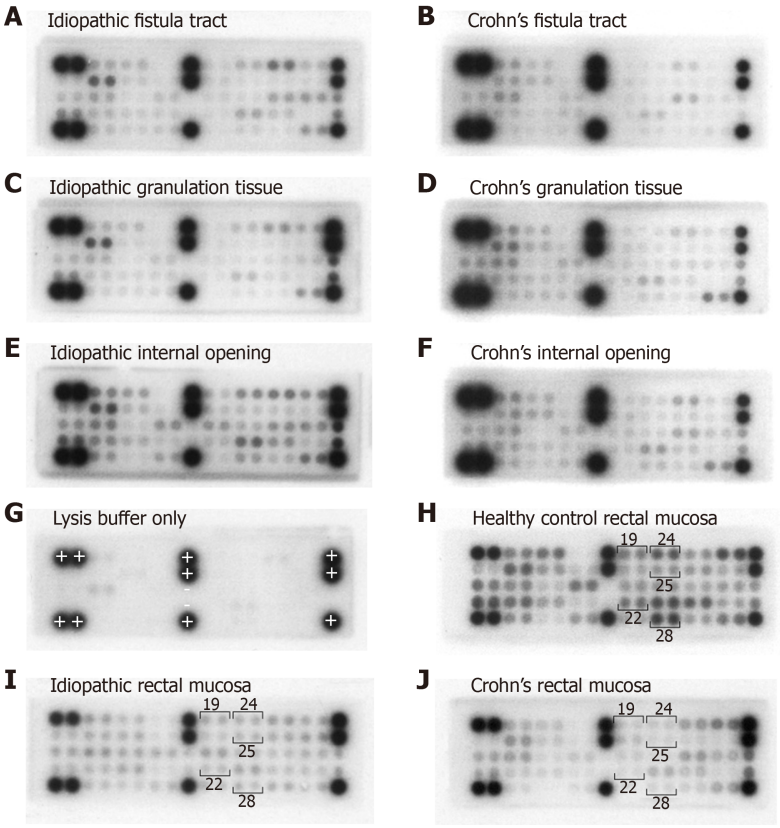

Figure 4 Example microarray images acquired to quantify the phosphorylation status of receptor tyrosine kinases in the cell lysates of the fresh tissue samples using the PathScan RTK Signaling Antibody Array, Chemiluminescent Readout (Cell Signaling Technology, Danvers, Massachusetts). Fistula tract samples from (A) idiopathic and (B) Crohn's disease patients. Granulation tissue samples from (C) idiopathic and (D) Crohn's disease patients. Internal opening samples from (E) idiopathic and (F) Crohn's disease patients. Control array containing lysis buffer only (G), showing an absent signal from all test spots and the negative control spots (-), and 100% signal from the positive control spots (+). Healthy control rectal mucosa (H) showing positive signal from numerous spots including EphA1 (19), EphB1 (22), EphB4 (24), Tyro3/Dtk (25) and VEGFR (28). Idiopathic rectal mucosa (I) showing significantly lower signal from the same highlighted spots. Crohnâs disease rectal mucosa (J), again showing significantly lower signal from the same highlighted spots.

RTK may be suppressed in these diseases.

Limitations

Only 13 patients were recruited to the CD group. Previous comparative studies have reported with similar sample sizes[10-13]. Preferential medical management in CD-related perianal fistulae could also have introduced selection bias, and referral patterns could have skewed the types of fistulae included to the more severe (especially in relation to the idiopathic group where many were tertiary referrals).

Techniques and assays were taken from similar experiments on intestinal mucosa published by our group[17]. The assays have not previously been used on fistula tract and granulation tissue, however their performance was likely to be similar as the cellular compositions of these tissues are similar.

The majority of the CD group were receiving immunomodulatory therapy, and half had received anti-TNF-α. This may have attenuated differences between the groups. However, the effect of anti-TNF-α therapy at the molecular level is unknown. One study found that mucosal TNF-α concentrations are unaltered by anti-TNF-α therapy[19]. Including only treatment-naïve patients would have mitigated this, but recruitment would have been difficult into given that most are primarily managed medically.

Conclusion

CD-related perianal fistulae may often be clinically more severe and complex. However, they do not substantially differ in their expression of a large panel of cytokines and phosphoproteins (Table 2).

Implications for future research

Table 2 Summary of comparisons between idiopathic and Crohn's disease perianal fistulae

	Similarities	Differences
PDAI		Significantly higher PDAI in Crohn's disease
EQ-5D-5L	Similar EQ VAS and EQ index	
Morphology	Similar distribution of types by Parks' classification	More multiple fistulae in Crohn's disease
	Similar prevalence of high fistulae and horseshoe extensions	Significantly more supralevator extensions, collections and rectal thickening in Crohn's disease
Cytokine concentrations	Similar concentrations of 27 cytokines at all four biopsy sites	Significantly higher IL-12p70 concentration at internal opening in Crohn's disease
	(EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, RANTES and VEGF)	Significantly lower IL-1RA/IL-1β ratio concentration at internal opening in Crohn's disease
Phosphoprotein concentrations	Similar levels of 39 phosphoproteins at the four specimen sites	
	(EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, FGFR1, FGFR3, FGFR4, InsR, IGF-IR, TrkA/NTRK1, TrkB/NTRK2, Met/HGFR, Ron/MST1R, Ret, ALK, PDGFR, c-Kit/SCFR, FLT3/Flk2, M-CSFR/CSF-1R, EphA1, EphA2, EphA3, EphB1, EphB3, EphB4, Tyro3/Dtk, Axl, Tie2/TEK, VEGFR2/KDR, Akt/PKB/Rac (at Thr308), Akt/PKB/Rac (at Ser473), p44/42 MAPK, S6 Ribosomal Protein, c-Abl, IRS-1, Zap-70, Src, Lck, Stat1 and Stat3)	

See supplementary material for cytokine and phosphoprotein names in full. PDAI: Perineal Disease Activity Index; EQ: EuroQol; VAS: Visual analogue score.

Despite the acknowledged limitations (particularly regarding sample size in the CD group and the fact that some CD patients were receiving immunomodulatory therapy), our data contributes to an emerging theory that idiopathic and CD-related perianal fistulae may not be as immunologically distinct as previously supposed. This line of reasoning opens the possibility that biological agents effective in CD-related perianal fistulae may also have a role in selected idiopathic perianal fistulae especially when recent randomised trial data have exposed the general limitations of surgery[3]. We acknowledge however that research is warranted.

ARTICLE HIGHLIGHTS

Research background

Perianal fistulae are common and cause significant physical and psychosocial morbidity. Current treatments come with a significant failure rate. Idiopathic perianal fistulae are thought to arise from a primary infection of an anal gland, which leads to penetrating suppuration and fistula formation. Crohn's disease (CD)-related perianal fistulae is thought to arise from altered inflammatory pathways within the mucosa.

Research motivation

The aetiology and pathophysiology of perianal fistulae is still unclear. A better understanding could lead to better treatments. Most research to date has assumed idiopathic and CD-related fistulae to be fundamentally different. However this assumption has never been tested.

Research objectives

We hypothesised that idiopathic and CD-related perianal fistulae are different and aimed to test this systematically by comparing their clinical phenotypes, cytokine and phosphoprotein profiles.

Research methods

We conducted a prospective cohort study within a university hospital. Sixty-one consecutive patients undergoing surgery for perianal fistula were recruited. Clinical data, pre- and post-operative Perineal Disease Activity Index (PDAI) and EQ-5D-5L scores were measured. Biopsies of the fistula tract, granulation tissue, internal opening mucosa and rectal mucosa were obtained at surgery. These were processed in our laboratory to measure 30 cytokines and 39 phosphoproteins. To our knowledge, this is the largest study to date to systematically compare idiopathic and CD-related perianal fistulae in a well-defined patient cohort.

Research results

The PDAI was significantly higher and complex pathoanatomy was more prevalent in the CD group, supporting the commonly-held belief that CD-related perianal fistulae are more severe and complex than idiopathic. IL-12p70 concentration at the internal opening was higher and the IL-1RA/IL-1β ratio was significantly lower at the internal opening in patients with CD. There were no significant differences between the groups for any other cytokine concentrations at the four specimen sites. There were also no significant differences in phosphoprotein levels between the patient groups at any specimen site.

Research conclusions

CD-related perianal fistulae are often clinically more severe and complex. However, they do not substantially differ in their expression of a large panel of cytokines and phosphoproteins.

Research perspectives

Our data contributes to an emerging theory that idiopathic and CD-related perianal fistulae may not be as immunologically distinct as previously supposed. This line of reasoning opens the possibility that biological agents effective in CD-related perianal fistulae may also have a role in selected idiopathic perianal fistulae especially when recent randomised trial data have exposed the general limitations of surgery. Further research is warranted.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Fecal lactoferrin accurately reflects mucosal inflammation in inflammatory bowel disease

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Author contributions: Rubio MG contributed to the study design, participated in the data collection and analysis, and drafted the manuscript. Amo-Mensah K and Gray JM participated in data collection. Nguyen VQ contributed to the study design and participated in the analysis. Nakat S reviewed all CT and MRI images. Grider D performed the histological analyses. Love K performed the statistical analyses. Boone JH contributed to the study design and drafting of the manuscript. Sorrentino D designed and supervised the study; helped with the drafting of the manuscript and revised the final version. All the authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Studies have demonstrated a potential role for fecal biomarkers such as fecal calprotectin (FC) and fecal lactoferrin (FL) in monitoring inflammatory bowel diseases (IBD) - Crohn's disease (CD) and ulcerative colitis (UC). However, their correlation to endoscopic scores, disease severity and affected intestinal surface has not been extensively investigated.

AIM

To correlate FL, and for comparison white blood cell (WBC) and C-reactive protein (CRP), with endoscopic scores, disease extent and location in CD and UC.

METHODS

Retrospective analysis in 188 patients who had FL, CRP and WBC determined within 30 d of endoscopy. Disease location, disease extent (number of intestinal segments involved), disease severity (determined by endoscopic scores), timing

of FL testing in relation to colonoscopy, as well as the use of effective fast acting medications (steroids and biologics) between colonoscopy and FL measurement, were recorded.

RESULTS

In 131 CD and 57 UC patients, both CRP and FL - but not WBC - distinguished disease severity (inactive, mild, moderate, severe). In patients receiving fast-acting (steroids or biologics) treatment in between FL and colonoscopy, FL showed a higher correlation to endoscopic scores when tested before *vs* after the procedure ($r = 0.596$, $P < 0.001$, *vs* $r = 0.285$, $P = 0.15$ for the Simple Endoscopic Score for CD; and $r = 0.402$, $P = 0.01$ *vs* $r = 0.054$ $P = 0.84$ for Disease Activity Index). Finally, FL was significantly correlated with the diseased mucosal surface (colon-ileocolon > small bowel) and the number of inflamed colon segments.

CONCLUSION

FL and CRP separated disease severity categories with FL showing lower discriminating *P*-values. FL showed a close correlation with the involved mucosal surface and with disease extent and was more closely correlated to endoscopy when determined before the procedure – this indicating that inflammatory activity changes associated with therapy might be rapidly reflected by FL levels. FL can accurately and timely characterize intestinal inflammation in IBD.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Fecal lactoferrin; C-reactive protein; White blood cell count; Mucosal inflammation

Core tip: Studies have demonstrated a potential role for fecal biomarkers such as fecal calprotectin and fecal lactoferrin (FL) in monitoring Crohn's disease (CD) and ulcerative colitis (UC). However, their correlation with disease burden (endoscopic scores/disease activity and disease extent) has not been extensively investigated. In our study FL separated disease severity categories based on endoscopic scores in both UC and CD patients. FL showed a close correlation with the diseased mucosal surface and with disease extent and was more closely correlated to endoscopy when determined before endoscopy. FL can accurately and timely represent intestinal inflammation in inflammatory bowel diseases.

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INTRODUCTION

Inflammatory bowel disease (IBD), both Crohn's disease (CD) and ulcerative colitis (UC), are on the rise worldwide, including Asia[1-3]. In the United States, 3.1 million people are reported to be affected[4]. The annual burden of IBD is extensive, with over 2.3 million physician visits, 180000 hospital admissions, and a cost of $6.3 billion in healthcare services[5-7]. One third of the annual cost of healthcare for IBD patients is classified as outpatient services, with the major components being endoscopy and pathology[7].

IBD activity has traditionally been monitored by the severity of clinical symptoms, using clinical scoring systems such as the Crohn's Disease Activity Index (CDAI) for CD and the clinical component of the Mayo score for UC[8]. However, these measures are subjective and correlate poorly with objective findings[9].

Endoscopy is a more objective parameter of disease activity than clinical symptoms[10,11], but it is expensive, invasive, and often unwelcomed by patients. A number of studies have shown that fecal biomarkers, specifically fecal lactoferrin (FL) and fecal calprotectin (FC), are effective indicators of mucosal inflammation and

retrospective study was exempted by the Carilion Institutional Board. All data was deidentified by using a unique code.

Conflict-of-interest statement: Sorrentino D has received consulting fees from Abbott/AbbVie, Schering-Plough, MSD, Janssen Research & Development, LLC., Centocor Inc., TechLab, Hoffmann-LaRoche, Giuliani, Schering-Plough, and Ferring; research grants from AbbVie, Janssen Research & Development, LLC, Schering-Plough, TechLab, Centocor, Takeda and serves in the Speakers Bureau of AbbVie and the National Faculty of Janssen. Rubio MG has received a Grant from Gilead. Nguyen VQ has received grant support from AbbVie Inc. James H Boone is a Senior Scientist at TechLab, Inc. The other authors have no conflicts of interest to declare.

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injury[8,13-16]. Fecal biomarkers have been shown to be inexpensive, noninvasive, and reproducible, and they have a strong potential for use in monitoring IBD[12]. Both FC and FL have shown similar success clinically, and levels may rise significantly before clinical relapse and may predict subsequent IBD flares[17]. FL is an iron-binding glycoprotein expressed by active neutrophils-the primary component of the active inflammatory response[18,19]. FL is stable at room temperature for weeks, resistant to proteolysis, and resilient to multiple freeze-thaw cycles[20,21].

In a recent meta-analysis evaluating the diagnostic accuracy of FL in assessing IBD activity Dai and colleagues[22] found that in ten studies comprising 773 IBD patients the pooled sensitivity and specificity values for assessing UC activity were 0.81 [95% confidence interval (CI): 0.64-0.92] and 0.82 (95%CI: 0.61-0.93), respectively. The pooled sensitivity and specificity values for assessing CD activity were 0.82 (95%CI: 0.73-0.88) and 0.71 (95%CI: 0.63-0.78), respectively.

FL appears to be equally useful in CD and UC, when active or inactive disease is present[23]. The current knowledge gap lies in an incomplete understanding of correlation between FL levels and mucosal inflammation, disease location and extent.

In this retrospective study we investigated the correlation between these parameters and FL levels and compared them to C-reactive protein (CRP) and white blood cell count (WBC), two widely used indicators of inflammation.

MATERIALS AND METHODS

Study population

This retrospective study enrolled patients seen in our Center from 2008 to 2018, diagnosed with UC or CD according to widely established criteria including histology[24] and were monitored as standard of care through the measurement of FL levels. To be enrolled in the study patients had to have a colonoscopy done within 30 d of the FL test.

All clinical and laboratory data were collected from the patients' EMR (EPIC) and endoscopy data from images and reports stored in the Olympus Endoscopy Suite Program (Endoworks 7.4). There was no direct patient involvement. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Carilion Clinic Ethical Committee. No patient consent was deemed necessary by the Ethical Committee.

Biomarker testing

FL stool test (normal range 0-7.24 μg/mL) was measured quantitatively by the LACTOFERRIN SCAN (TECHLAB, Blacksburg, VA, United States), an enzyme-linked immunosorbent assay (ELISA). Serum measurements of CRP and WBC used established clinical laboratory methods.

Assessment of disease activity

For each IBD patient in this study, we reviewed endoscopic pictures and procedure reports to determine the severity of inflammation, mucosal injury, and disease location (see "Endoscopic scoring and disease extent" below). In CD patients, disease location was also based on magnetic resonance imaging (MRI) or computed tomography (CT) scan image results. The imaging reviewer was blinded to these patients FL levels at the time of the evaluation.

Endoscopic scoring and disease extent

The Simple Endoscopic Score for CD (SES-CD) (Supplementary Table 1) and the endoscopic component of the Mayo Clinical score [Disease Activity Index (DAI)] (Supplementary Table 2) for UC were utilized to measure endoscopic disease activity[9]. The SES-CD defines: Remission (score 0-2), mild inflammation (score 3-6), moderate inflammation (score 7-15), and severe inflammation (score ≥ 16). The endoscopic component of the DAI defines: Normal or inactive disease (0), mild disease (1), moderate disease (2), and severe disease (3). Since the DAI does not include a measure of disease extent, we estimated this parameter using a simple scoring system providing one point for each colonic segment (rectum, sigmoid, descending, transverse, ascending, cecum, ileo-cecal valve) demonstrating signs of disease. A score of 0 points indicates no disease and a score of 7 points indicates pan-colitis.

Statistical analysis

Descriptive statistics were used for patients' characteristics. Analysis of variance (ANOVA) was used on natural log-transformed values of FL, CRP and WBC to determine whether median values significantly varied according to SES-CD and DAI

scores. The values were natural log transformed for use in the analysis to address the non-normality of the distributions of the biomarkers, resulting in a comparison of median values on the original scale. Post-hoc pairwise comparisons using the method of least significant differences were used to determine which levels of SES-CD and DAI significantly differed in median biomarker values from other levels. Non-parametric Spearman correlations (due to non-normality of variables) were used to quantify and test the correlation of numeric SES-CD and DAI with FL levels, separately for patients whose FL levels were determined pre- and post-colonoscopy. To contrast FL levels with the number of colonic segments involved the Kruskal Wallis test followed by pairwise Mann-Whitney comparisons was used. The 0.05 level of significance was used for all statistical tests. The statistical review of the study was performed by a biostatistician (Love K).

RESULTS

Patient population

Using the inclusion criteria outlined in methods and data search spanning from 2008 to 2018 we identified a total of 188 IBD patients followed at Carilion Clinic IBD Center. The colonoscopy procedures were performed for standard of care indications (surveillance, monitoring or disease staging). Patient characteristics and clinical data are shown in Table 1. Overall, 59% of patients were female and 70% had CD. DAI scoring in UC patients showed mild disease in 19%, moderate disease in 48% and severe disease in 33%. Of the CD patients scored by SES-CD using the endoscopy results 10% had inactive disease, 22% had mild disease, 28% had moderate disease and 40% had severe CD. A total of 63% of UC patients had disease extending proximal to the splenic flexure (with 2/3 of patients having pancolitis), 30% had disease distal to the splenic flexure and 7% had proctitis. Of the CD patients 34% had ileal disease, 22% had ileocolonic disease and 44% had colonic involvement. In the CD group, 18% of CD patients had stricturing disease with 73% bearing non-stricturing, non-penetrating disease.

Biomarker levels according to disease severity

The biomarkers FL, CRP and WBC were evaluated as potential indicators of IBD disease severity as determined by endoscopic scores. In CD patients, FL median levels showed a significant difference for inactive (20 μg/g) *vs* mild (102 μg/g), inactive *vs* moderate (104 μg/g), mild *vs* severe (762 μg/g) and moderate *vs* severe activity. CRP median levels were significantly different for inactive (0.41 mg/dL) *vs* severe (2.43 mg/dL), and mild (0.87 mg/dL) *vs* severe activity (Figure 1). In UC patients, FL separated mild (56 μg/g) *vs* moderate (427 μg/g), and mild *vs* severe (766 μg/g) cases. CRP was significantly different between mild (0.35 mg/dL) *vs* severe (1.60 mg/dL), and moderate (0.55 mg/dL) *vs* severe cases (Figure 2). WBC median levels were similar across all categories for both CD and UC. Comparisons contrasting the accuracy in discriminating different disease activities showed less significant *P* values for CRP than for FL.

Correlation of biomarkers with endoscopic scores

Biomarker concentrations were compared to individual endoscopic scores for CD and UC patients. Overall, both FL and CRP showed the highest Spearman correlations to SES-CD and DAI scores for all assessed patients (Table 2). WBC had a very weak correlation to both the SES-CD and DAI scores. Next, we selected patients (n = 139) in whom fast acting therapy (steroids and biologics) was successfully initiated in between FL and colonoscopy. When patients were stratified in two groups according to the timing of FL testing (Table 3) FL showed a higher correlation to SES-CD and DAI when it had been tested before the procedure compared to when it had been tested after the procedure: for SES-CD r = 0.596, P < 0.001, *vs* r = 0.285, P = 0.149 (n = 58 and 27, respectively); for DAI r = 0.402, P = 0.012, *vs* r = 0.054, P = 0.842 (n = 38 and 16, respectively).

Biomarker levels according to disease location and extent of colonic disease

Of the 188 IBD patients included in the study, a total of 114 patients had colonic disease, 45 had isolated small bowel disease and 29 patients had both colonic and small bowel disease. FL, CRP and WBC levels relative to disease location were determined in 188, 152 and 153 patients, respectively. FL levels were significantly higher in patients with colonic and combined colonic and small bowel disease compared to patients with small bowel disease only (Figure 3A) with 69% of the latter patients having elevated (> 7.25 μg/g) levels of FL. CRP showed a similar trend with elevated levels being present in 44% of patients with small bowel disease (Figure 3B).

Table 1 Patient characteristics, *n* (%)

Characteristics		UC (*n* = 57)	CD (*n* = 131)
Gender	Female	27 (47)	83 (63)
	Male	30 (53)	48 (37)
Disease duration (yr)	0-5	37 (65)	72 (55)
	6 to 10	9 (16)	16 (12)
	> 10	11 (19)	43 (33)
Smoking	Current	1 (2)	32 (25)
	Former	29 (51)	37 (28)
	Never	27 (47)	62 (47)
Biomarkers	Lactoferrin μg/g	448 (0; 8467)	95 (0; 9351)
Median (min; max)	CRP mg/dL	0.54 (0.4; 17.3)	1.15 (0.4; 21.1)
	WBC (K/μL)	8.2 (4.0; 29.9)	7.5 (3.6; 26.4)
Montreal age (yr)	A1 < 16	0 (0)	4 (3)
	A2 17-40	29 (51)	47 (36)
	A3 > 40	28 (49)	80 (61)
Montreal class UC	E1 proctitis	4 (7)	
	E2 distal	17 (30)	
	E3 extensive	36 (63)	
Disease activity by DAI	Mild (1)	11 (19)	
	Moderate (2)	27 (48)	
	Severe (3)	19 (33)	
Montreal class CD	L1 ileal		45 (34)
	L2 colonic		57 (44)
	L3 ileocolonic		29 (22)
	L4 isolated upper		0 (0)
	B1 nonstricturing		95 (73)
	B2 stricturing		24 (18)
	B3 penetrating		12 (9)
	P perianal		4 (3)
Disease activity by SES-CD	Inactive (0-2)		9 (10)
	Mild (3-6)		20 (22)
	Moderate (7-15)		26 (28)
	Severe (≥ 16)		37 (40)

WBC was significantly different only between patients with colonic and those with combined colonic and small bowel disease (Figure 3C). In patients with colonic disease, FL levels were associated with the number of inflamed colon segments (Table 4). Patients with 0-1 inflamed segments had a median FL level of 8 μg/g. Patients with 6 to 7 inflamed segments had a median level of 789 μg/g. Overall the association was highly significant (Kruskal Wallis test: $P < 0.001$).

DISCUSSION

Colonoscopy is currently considered the standard test to diagnose and monitor IBD. However, this procedure is invasive, costly and requires sedation and an inconvenient preparation. Over the last two decades, fecal biomarkers such as FL and FC have emerged as potential substitutes of endoscopy. The advantages of fecal biomarkers are that samples (feces) are easy to obtain, can be collected at home, can be serially obtained, and are relatively easy to analyze. This allows patients to regularly monitor their disease without the need to see the clinician, by simply taking the stool sample to the laboratory. Hence, fecal biomarkers in principle offer a convenient, non-invasive and low-cost option for disease monitoring. How to best use these indicators to manage the disease is currently a subject of study and the initial results for FC are encouraging[25]. Less studies are available for FL. Gisbert *et al*[17] measured FL and FC in their study cohort. For FL they found that elevated values correlated with an

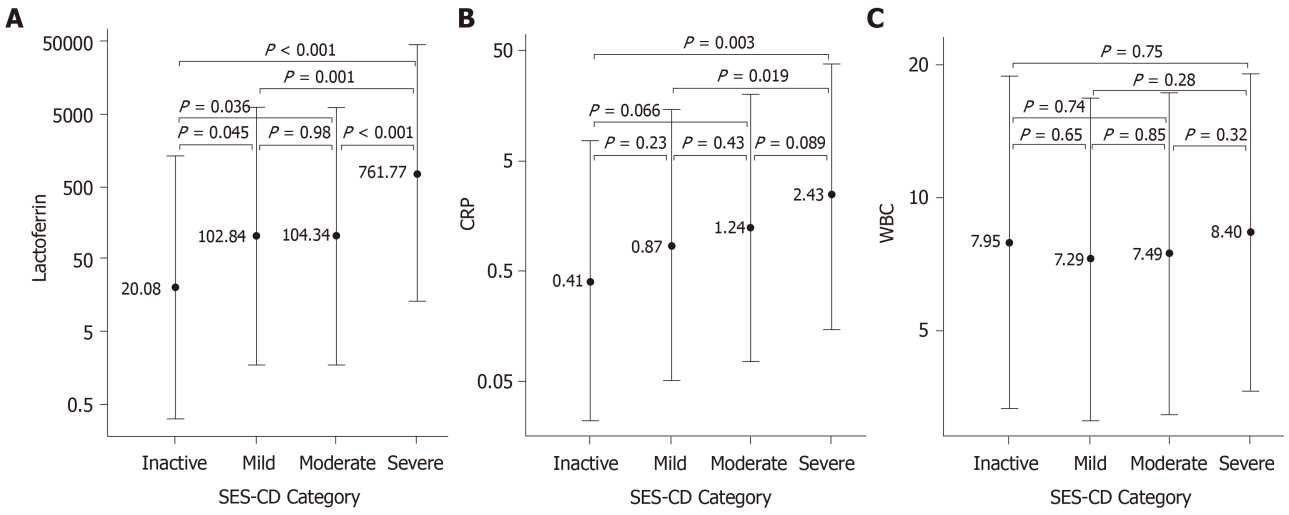

Figure 1 Biomarker levels according to disease severity measured by Simple Endoscopic Score for Crohn's disease in Crohn's disease patients. Analysis of variance was used on natural log-transformed values of fecal lactoferrin, white blood cell and C-reactive protein to determine whether median values significantly varied according to Simple Endoscopic Score for Crohn's disease scores. Fecal lactoferrin (A) and, less, C-reactive protein (B) showed correlation with Simple Endoscopic Score for Crohn's disease categories. WBC median values were similar across all categories (C). SES-CD: Simple Endoscopic Score for Crohn's disease; WBC: White blood cell; CRP: C-reactive protein.

increased risk of relapse (25% risk with a positive result *vs* 10% with a negative result, $P < 0.05$), and predicted relapse with a sensitivity of 62% and a specificity of 65%. Siponnen and colleagues reported a correlation of FL with SES-CD and with colonic histology in CD colitis, but not in small bowel disease[26]. In a recent review and metanalysis Dai *et al*[22] reported the results of 10 studies which used a prospective design and enrolled patients with diagnosed IBD. Overall the analysis revealed high discrimination for assessing UC and CD activity with FL concentration, with the diagnostic performance of the FL assay being apparently superior in UC patients. The authors concluded that FL is an inexpensive and useful screening marker with high sensitivity and modest specificity for assessing IBD activity[22].

In this retrospective study we investigated the correlation between FL levels and mucosal inflammation as well as disease location and extent, as assessed by endoscopy and histology in both UC and CD patients. For comparison we also tested the potential correlation of disease activity and location/extent with CRP and WBC – two blood markers also known to be elevated in inflammatory states. We only included patients who underwent endoscopy within 30 d of markers measurement. The patient population was representative of the entire spectrum of disease severity.

The results show a positive, significant correlation of FL and CRP with SES-CD and DAI. Such correlation was not seen for WBC. When stratifying patients for levels of disease activity FL was able to separate inactive *vs* mild, inactive *vs* moderate, mild *vs* severe and moderate *vs* severe activity in CD. In UC patients, FL separated mild *vs* moderate, and mild *vs* severe disease activity. Importantly, in patients with exclusive colonic involvement FL levels increased with the number of colonic segments involved. FL levels were also elevated in 69% of active small bowel-only CD patients with median FL levels significantly lower compared to patients with colonic-only disease and combined colonic and small bowel disease (26 μg/g *vs* 304 μg/g *vs* 197 μg/g respectively). This finding suggests that low FL levels might be associated with small bowel disease activity but with minimal, if any, colonic disease activity. This could be related to the different surface area of the two intestinal tracts - whereby a small extent of disease activity is relatively more significant in the small bowel than in the colon. However, the precise explanation for this finding (as well as the establishment of a FL cut-off level for inflammation in the colon *vs* small bowel) must await a dedicated prospective study. Nevertheless, we show here that FL is a mostly reliable indicator of small bowel disease. This finding disagrees with that of Sipponen *et al*[26], who did not find correlation of fecal markers with small bowel disease. However, those authors only used colonoscopy to estimate the disease presence/extent in the small bowel whereas we also relied on imaging. Another potential confounding factor in determining the accuracy of fecal markers in small bowel disease is the unclear contribution of inflammation in the deeper layers of the gut wall[27]. Regardless, the finding that FL levels are correlated with the disease extent is an important one because it does indicate that this marker might be an accurate indicator of the total disease burden - the product of severity times extent[28] - and

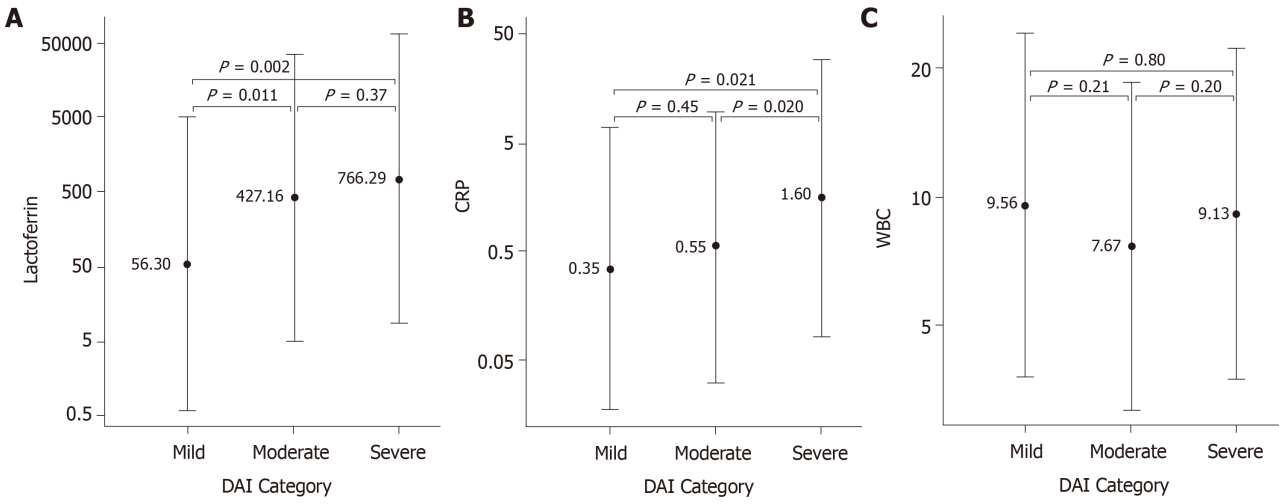

Figure 2 Biomarker levels according to disease severity measured by Disease Activity Index in ulcerative colitis patients. Analysis of variance was used on natural log-transformed values of fecal lactoferrin, white blood cell and C-reactive protein to determine whether median values significantly varied according to Disease Activity Index scores. Fecal lactoferrin (A) and C-reactive protein (B) showed correlation with Disease Activity Index categories. WBC median values were similar across all categories (C). DAI: Disease Activity Index; WBC: White blood cell; CRP: C-reactive protein.

makes it a potential candidate as an ideal therapeutic target, as already indirectly shown by others for FC[25].

Another interesting finding of our study is that FL levels obtained before colonoscopy had a better correlation with SES-CD and DAI than levels measured after the colonoscopy in patients given effective, fast acting medications (steroids and biologics) in between marker determination and the procedure. The most likely explanation of this observation is that FL is a timely indicator of changes in disease activity after therapy. FL concentration in feces is proportional to neutrophil translocation to the mucosa of the GI tract – a process that is quickly modulated by the activity of the inflammatory process[19]. Replacement of the dead epithelial cells on the other hand might be more lengthy[29]. In principle, such timeliness of FL reaction to changes in the mucosa inflammatory activity in IBD could be exploited to monitor treatment response in a number of clinical scenarios.

Our study has some obvious limitations. Firstly, it is a retrospective study. However, the data were retrieved from a single EMR and included detailed and uniform information on patients' clinical status, disease features and treatment. Although FL was not tested for research purposes the timing and accuracy of data acquisition would not have been different in a prospective study. Secondly, our study is a single center study. As such it is possible that it might reflect the investigators' and the institution standard of practice as they relate to several aspects of this study. However, we applied the strictest and most objective criteria to select our patients' population – according to widely used standards. Furthermore, the size of our patient population and the representation of the spectrum of disease activity and extent are well above the average of studies focused on FL[22].

Further validation of our findings in larger scale and prospective studies might confirm that fecal markers of inflammation are accurate and inexpensive indicators of disease activity in IBD, can be used in a number of clinical scenarios and should become part of the standard armamentarium of the practicing gastroenterologist, especially in the United States where their use still lags behind most other Western countries[30].

Table 2 Correlation of Simple Endoscopic Score for Crohn's disease and Disease Activity Index and biomarkers

Disease severity score	Disease marker	Spearman correlation
SES-CD	FL	0.563[b]
	CRP	0.433[b]
	WBC	0.152
DAI	FL	0.330[a]
	CRP	0.410[b]
	WBC	0.099

[a]$P < 0.05$,
[b]$P < 0.01$. DAI: Disease Activity Index; SES-CD: Simple Endoscopic Score for Crohn's disease; WBC: White blood cell; CRP: C-reactive protein; FL: Fecal lactoferrin.

Table 3 Spearman correlation of fecal lactoferrin with Simple Endoscopic Score for Crohn's disease and Disease Activity Index according to timing of tests

Endoscopic scores	Analysis	FL pre colonoscopy	FL post colonoscopy
SES-CD	Correlation Coefficient	0.596[b]	0.285
	N	58	27
DAI	Correlation Coefficient	0.402[a]	0.054
	N	38	16

[a]$P < 0.05$,
[b]$P < 0.01$. DAI: Disease Activity Index; SES-CD: Simple Endoscopic Score for Crohn's disease; FL: Fecal lactoferrin.

Table 4 Median fecal lactoferrin *vs* extent of colonic disease

Number of inflamed colon segments	Number of patients	Median FL (μg/g)
0-1	37	8
2-3	42	91.5
4-5	21	302
6-7	43	789

Kruskal Wallis test $P < 0.001$. FL: Fecal lactoferrin.

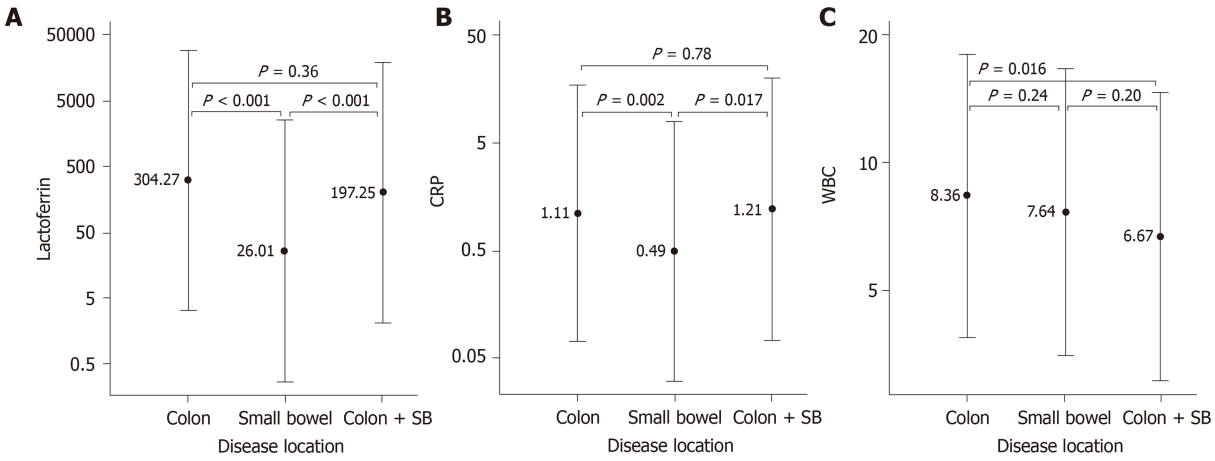

Figure 3 Biomarker levels according to disease location. Analysis of variance was used on natural log-transformed values of fecal lactoferrin, white blood cell and C-reactive protein to determine whether median values significantly varied according to disease location. Fecal lactoferrin levels (A) were significantly higher in patients with colonic and combined colonic and small bowel disease compared to patients with small bowel disease only. C-reactive protein (B) showed a similar trend while white blood cell (C) was significantly different only between patients with colonic and those with combined colonic and small bowel disease. WBC: White blood cell; CRP: C-reactive protein; SB: Small bowel.

ARTICLE HIGHLIGHTS

Research background

Studies have demonstrated a potential role for fecal biomarkers such as fecal calprotectin (FC) and fecal lactoferrin (FL) in monitoring inflammatory bowel diseases (IBD) – both Crohn's disease (CD) and ulcerative colitis (UC). However, their correlation to endoscopic scores, disease severity and affected intestinal surface has not been extensively investigated.

Research motivation

Achieving a better understanding of the role of fecal markers for the evaluation and management of IBD patients.

Research objectives

To correlate FL, and for comparison white blood cell (WBC) and C-reactive protein (CRP), with endoscopic scores, disease extent and location in CD and UC.

Research methods

Retrospective analysis in 188 patients who had FL, WBC and CRP determined within 30 d of endoscopy. Disease location, disease extent (number of intestinal segments involved), disease severity [determined by endoscopic scores: Simple Endoscopic Score for CD (SES-CD) and the endoscopic component of the Mayo Clinical score/Disease Activity Index (DAI)], timing of FL testing in relation to colonoscopy, as well as the use of effective fast acting medications (steroids and biologics) between colonoscopy and FL measurement, were recorded.

Research results

In 131 CD and 57 UC patients, both CRP and FL - but not WBC - distinguished disease severity (inactive, mild, moderate, severe). In patients receiving fast-acting treatment (steroids or biologics) in between FL measurement and colonoscopy, FL showed a higher correlation to endoscopic scores when tested before *vs* after the procedure ($r = 0.596$, $P < 0.001$ *vs* $r = 0.285$, $P = 0.15$, for SES-CD; and $r = 0.402$, $P = 0.01$, *vs* $r = 0.054$, $P = 0.84$ for DAI). Finally, FL was significantly correlated with the diseased mucosal surface (colon-ileocolon > small bowel) and the number of inflamed colon segments. FL and CRP separated disease severity categories. FL showed a close correlation with the involved mucosal surface and with disease extent and was more closely correlated to endoscopy when determined before the procedure – this indicating that inflammatory activity changes associated with therapy might be rapidly reflected by FL levels.

Research conclusions

The results show a positive, significant correlation of FL and CRP with SES-CD and DAI. FL showed a close correlation with the diseased mucosal surface and with disease extent and was more closely correlated to endoscopy when determined before endoscopy. FL can accurately and timely represent intestinal inflammation in IBD.

Research perspectives

Further validation of our findings in large scale and prospective studies might confirm that fecal markers of inflammation are accurate and inexpensive indicators of disease activity in IBD, can be used in a number of clinical scenarios and should become part of the standard armamentarium of the practicing gastroenterologist.

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