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EDITORIAL

Emerging roles of myeloid derived suppressor cells in hepatic inflammation and fibrosis

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Abstract

Myeloid derived suppressor cells (MDSC) are a heterogeneous population of immune cells that are potent suppressors of immune responses. MDSC emerge in various compartments in the body, such as blood, bone marrow or spleen, especially in conditions of cancer, infections or inflammation. MDSC usually express CD11b, CD33, and low levels of human leukocyte antigen-DR in humans or CD11b and Gr1 (Ly6C/G) in mice, and they can be further divided into granulocytic or monocytic MDSC. The liver is an important organ for MDSC induction and accumulation in hepatic as well as extrahepatic diseases. Different hepatic cells, especially hepatic stellate cells, as well as liver-derived soluble factors, including hepatocyte growth factor and acute phase proteins (SAA, KC), can promote the differentiation of MDSC from myeloid cells. Importantly, hepatic myeloid cells like neutrophils, monocytes and macrophages fulfill essential roles in acute and chronic liver diseases. Recent data from patients with liver diseases and animal models linked MDSC to the pathogenesis of hepatic inflammation, fibrosis and hepatocellular carcinoma (HCC). In settings of acute hepatitis, MDSC can limit immunogenic T cell responses and subsequent tissue injury. In patients with chronic hepatitis C, MDSC increase and may favor viral persistence. Animal models of chronic liver injury, however, have not yet conclusively clarified the involvement of MDSC for hepatic fibrosis. In human HCC and mouse models of liver cancer, MDSC are induced in the tumor environment and suppress anti-tumoral immune responses. Thus, the liver is a primary site of MDSC *in vivo*, and modulating MDSC functionality might represent a promising novel therapeutic target for liver diseases.

Key words: Myeloid derived suppressor cells; Interleukin-10; Treg; Liver cirrhosis; Macrophage; Hepatitis C virus

Core tip: Myeloid derived suppressor cells (MDSC) are a heterogeneous population of immune-suppressive cells with important roles during inflammation, infection and cancer. The liver is a primary site for MDSC induction

and accumulation, and recent studies linked these cells to the pathogenesis of hepatic inflammation, fibrosis and hepatocellular carcinoma. MDSC can limit tissue injury during acute hepatitis, while they may favor viral persistence in chronic hepatitis. MDSC are also induced during development of liver cancer and suppress anti-tumoral immunity, but their involvement in hepatic fibrosis is less clear. Thus, modulating MDSC functionality might represent a promising novel therapeutic target for liver diseases.

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INTRODUCTION

Myeloid-derived suppressor cells (MDSC) are a heterogeneous cell population of myeloid origin originally described in tumor-bearing hosts[1] that are also induced under various inflammatory conditions - including sepsis[2], hepatitis[3,4] and viral infections[5-7]. MDSC regulate immune responses by potently suppressing T cell function[8]; although these T cell suppressive activities have been functionally linked to tumor progression or evasion from immune responses, the exact roles of MDSC appear to be context-dependent and vary between infectious, autoimmune or malignant diseases. MDSC are usually identified as $CD11b^+$ $CD33^+$ $HLA\text{-}DR^{low}$ cells in humans and $CD11b^+$ $Gr1^+$ cells in mice[9]. However, a specific marker for MDSC has not been described so far, which can make identification of these cells difficult as all those surface molecules are shared with other myeloid cell types such as neutrophils, monocytes or myeloid dendritic cells. Therefore, the most reliable feature to distinguish MDSC from other myeloid cells seems to be their suppressive function.

MDSC consist of at least two major subpopulations that are termed monocytic MDSC (mMDSC) and granulocytic MDSC (gMDSC) according to their side scatter (SSC) profile and Gr1 (Ly6C/G) expression in mice[10]. Whereas murine mMDSC have a low SSC profile and are $Ly6C^{hi}$ $Ly6G^-$, gMDSC are $Ly6C^{lo}$ $Ly6G^{hi}$ and show a higher SSC profile. In humans, CD14 and CD15 have been suggested as markers for mMDSC and gMDSC, respectively, but further investigation is needed to verify this hypothesis[11]. The two subsets seem to differ in their suppressive capacity and functional mechanism(s) depending on the disease studied.

As MDSC are heterogeneous myeloid cells with immune suppressive functions, several mechanisms of T cell suppression have been described. These mechanisms include L-arginine depletion by the enzymes arginase 1 (Arg1) or inducible nitric oxide synthase (iNOS) and generation of reactive oxygen species (ROS)[8,10,12]. Furthermore, MDSC have also been shown to secrete anti-inflammatory cytokines like IL-10[13]. Again, the suppressive mechanisms used by the different subsets as well as the requirement of cell-cell-contacts *vs* secretion of soluble factors seem to be highly dependent on the underlying pathology (Table 1). A recent study on the development of murine MDSC suggested that the two subsets depend on the expression of distinct anti-apoptotic proteins and that T cell suppressive functions are restricted to the mMDSC subset[14].

THE LIVER AS A SITE OF MDSC ACCUMULATION AND INDUCTION

The liver has been shown to be a site of MDSC accumulation, and this seems to apply to hepatic and also to extrahepatic diseases. Different hepatic cell types as well as liver-derived soluble factors have been implicated in the recruitment and differentiation of MDSC under various conditions (Figure 1). In tumor-bearing mice with various types of cancer - including breast, lung and skin cancer - MDSC numbers increased in the liver irrespective of whether the mice had tumor manifestation in the liver, namely hepatic metastasis, or not[15]. Furthermore, adoptively transferred MDSC homed to livers and spleens of tumor-bearing mice in a comparable fashion. Ilkovitch *et al*[15] could show that this increase in hepatic MDSC is at least in part due to elevated levels of GM-CSF, a hematopoietic growth factor produced by many different types of tumors and associated with splenic accumulation of MDSC.

Additionally, hepatic stellate cells (HSC), a cell type associated with various immune-modulatory functions[16], have been shown to induce MDSC from myeloid cells in mice and men. Primary human HSC were able to induce differentiation of MDSC from PBMC *in vitro*[17]. This induction was dependent on direct cell-cell contacts as well as on the expression of CD44 by HSC and led to generation of $CD14^+$ $HLA\text{-}DR^{lo}$ cells able to suppress T cell responses in an arginase 1-dependent manner. Similarly, murine hepatic stellate cells were proven to induce $CD11b^+Gr1^+$ MDSC from bone marrow-derived cells[18,19]. However, this induction seems to be mediated by soluble factors rather than cell-cell contact. Chou *et al*[18] implicated a critical role for IFNγ signaling in HSC, and an additional study from the same group showed that MDSC induction was mediated by complement component C3 released by HSC[19]. In addition, both studies demonstrated that HSC could also induce MDSC *in vivo* in the context of islet cell transplantation and therefore contribute to allograft survival.

Furthermore, liver-derived soluble factors can also promote the generation of MDSC (Figure 1). Human mesenchymal stromal cells and an osteosarcoma cell line are able to induce the expansion of $CD11b^+$ $CD33^+$ $CD14^-$ MDSC from peripheral blood leukocytes *in vitro*, an effect that is mediated by hepatocyte growth

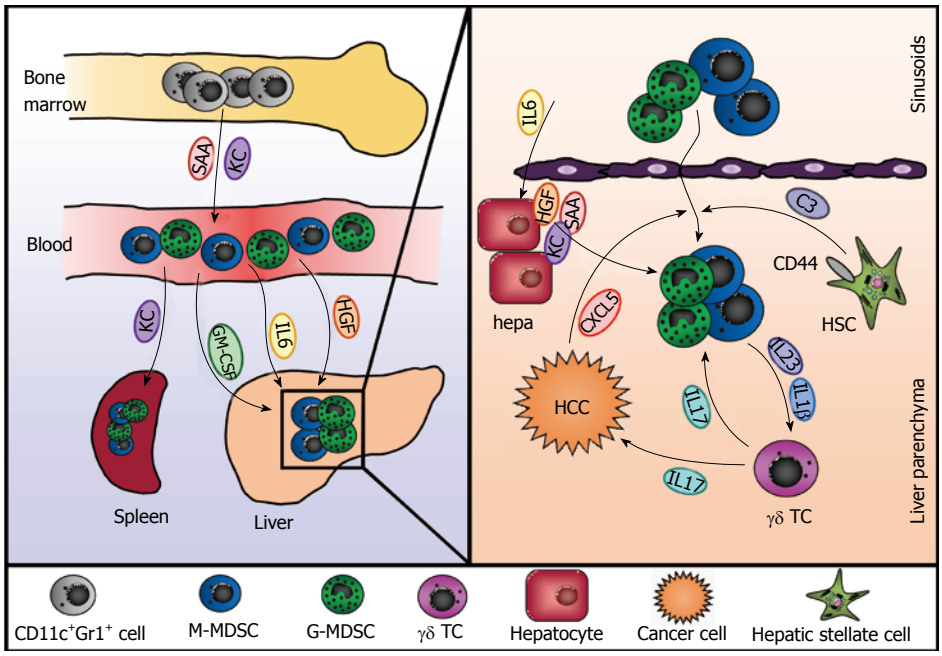

Figure 1 Myeloid derived suppressor cells in liver disease. Left: Myeloid derived suppressor cells (MDSC) accumulate during infectious, inflammatory or malignant diseases in several compartments of the body, including the liver. MDSC potently suppress immunogenic T cell responses, which is also relevant for liver diseases such as hepatic inflammation, fibrosis or HCC. Right: The induction of monocytic (mMDSC) or granulocytic (gMDSC) MDSC in the liver is promoted by different cell types in the liver *via* cell-cell-contact dependent mechanisms (*e.g.*, CD44) as well as *via* various soluble mediators. Details are provided in the main text. GM-CSF: Granulocyte-macrophage colony-stimulating factor; HCC: Hepatocellular carcinoma; hepa: Hepatocyte; HGF: Hepatocyte growth factor; HSC: Hepatic stellate cell; IL: Interleukin; SAA: Serum amyloid A; TC: T cell.

factor (HGF) and its receptor c-Met[20]. Since the liver usually harbors high levels of HGF this might be an explanation for the high numbers of MDSC present in the liver even under steady state conditions. Indeed, inhibition of the HGF/c-Met pathway in mice led to a significant reduction in hepatic but not splenic MDSC[20]. In the context of polymicrobial sepsis in mice hepatic acute-phase proteins play a critical role for controlling the inflammatory reaction to infection. Both serum amyloid A (SAA) and the chemokine CXCL1/KC work synergistically to mobilize MDSC from the bone marrow and induce their accumulation in the spleen[2]. Mice lacking the production of acute phase proteins due to the deletion of the IL-6 cytokine family receptor gp130 in hepatocytes showed less accumulation of MDSC and increased mortality during sepsis, which could be reversed by adoptive transfer of MDSC or administration of recombinant SAA and KC[2]. Consistently, the ectopic expression of IL-6 in the liver induced accumulation of MDSC in liver and spleen, which protected mice from $CD8^+$ T cell-mediated liver injury[21].

Another factor that may contribute to for the accumulation of MDSC in the liver is activation of inflammasomes, proteolytic complexes activated by pattern recognition receptors (PRR), and resulting in the production of IL-1β and IL-18. In murine cancer models activation of the Nlrp3 inflammasome has been associated with the accumulation of MDSC and suppression of anti-tumor immune responses[22,23]. This may also apply to liver diseases as inflammasome activation is important in a wide range of conditions[24,25]. Chronic human liver diseases are often associated with changes in the intestinal microbiome with the resulting inflammation leading to disruption and enhanced permeability of the intestinal epithelial barrier[26,27]. This enables the translocation of microbial products, which can travel to the liver *via* the portal vein and activate the inflammasome complex through PRRs. So far, this process has mainly been described for liver macrophages[28], but considering what has been observed for tumor-associated MDSC, inflammasome activation might also induce accumulation of hepatic MDSC.

MDSC IN THE REGULATION OF HUMAN LIVER DISEASES

While the above mentioned data demonstrated that the liver is an important site of MDSC induction for extrahepatic infections and cancer, more recent data implied hepatic MDSC as essential regulators of liver diseases as well. Several studies have concordantly reported that patients with hepatocellular carcinoma (HCC) or chronic hepatitis C virus (HCV) infection show increased frequencies of MDSC in the peripheral blood[6,7,29-32]. Human MDSC in HCC patients are mainly $CD14^+$ $HLA\text{-}DR^{-/low}$ and able to inhibit T cell proliferation in an arginase dependent manner[29]. Furthermore, these cells induce a regulatory phenotype in $CD4^+$ T cells and inhibit natural killer (NK) cell function *in vitro*[29,33]. Likewise, MDSC in the blood of patients

Table 1 Functional role of myeloid derived suppressor cells in the regulation of human and murine liver diseases

Species	Type of disease	Surface phenotype	Function of MDSC	Mechanism	Ref.
Human	Chronic HCV infection	$CD11b^+$ $HLA\text{-}DR^{lo}$ $CD33^+$ $CD14^+$	Inhibition of T cell proliferation and IFNγ production	Arginase1	[6]
Human	HCV-infected hepatocytes	$CD11b^{+/lo}$ $HLA\text{-}DR^{lo/-}$ $CD33^+$ $CD14^+$	Inhibition of T cell cytokine production	ROS Cell-cell-contact	[7]
Human	HCC	$CD11b^+$ $HLA\text{-}DR^-$ $CD33^+$ $CD14^-$	Long-lasting inhibition of effector T cells		[22-30]
Human	HCC	$HLA\text{-}DR^{lo/-}$ $CD14^+$	Inhibition of natural killer cells	Cell-cell-contact NKp30	[33]
Human	HCC	$HLA\text{-}DR^{lo/-}$ $CD14^+$	Induction of Treg and inhibition of effector T cells	Arginase	[29]
Mouse	CCl_4-mediated fibrosis	$CD11b^+Ly6G^-Ly6C^{hi}F4/80^+$ $CD11b^+Ly6G^+Ly6C^{lo}F4/80^-$	Amelioration of fibrosis through inhibition of HSC	IL-10 production	[13]
Mouse	Th1-mediated inflammation	$CD11b^+Ly6G^-Ly6C^{hi}$ $CD11b^+Ly6G^+Ly6C^{lo}$	Inhibition of T cell proliferation ($CD4^+$ and $CD8^+$)	iNOS cell-cell-contact	[48]
Mouse	Sepsis	$CD11b^+Gr1^+$	Inhibition of IL-12 and induction of IL-10 release by macrophages	Cell-cell-contact	[2]
Mouse	Immune-mediated hepatitis	$CD11b^+Ly6G^{lo}Ly6C^{hi}$ $CD11b^+Ly6G^+Ly6C^{lo}$	Suppression of $CD4^+$ T cell proliferation	iNOS	[46,47]
Mouse	ConA-mediated hepatitis	$CD11b^+Ly6G^-Ly6C^+$ $CD11b^+Ly6G^+Ly6C^{+(int)}$	Protection against liver injury through inhibition of T cells	Arginase	[4]
Mouse	ConA/LPS-mediated hepatitis	$CD11b^+Ly6G^{lo}Ly6C^{hi}$ $CD11b^+Ly6G^{hi}Ly6C^{int}$	Suppression of $CD4^+$ T cell proliferation and cytokine production	iNOS cell-cell-contact	[3,45]
Mouse	CTL-mediated liver injury	$CD11b^+Gr1^+$	Suppression of CTL proliferation and IFNγ production		[21]
Mouse	HBV (transgenics)	$CD11b^+Gr1^+$	Suppression of HBV-specific CTL	Arginase iNOS	[5]
Mouse	HCC/primary liver tumors	$CD11b^+Gr1^+$	Suppression of anti-tumor CTL		[35,36,38]
Mouse	Gastrointestinal cancer with liver metastasis	$CD11b^+Gr1^{+/int}$	Inhibition of T cell proliferation and tumor cell lysis		[40]

ConA: Concanavalin A; CTL: Cytotoxic T lymphocyte; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; IFN: Interferon; IL: Interleukin; iNOS: Inducible nitric oxide synthase; ROS: Reactive oxygen species; Treg: Regulatory T cell; MDSC: Myeloid derived suppressor cells.

with chronic HCV were shown to be $CD11b^+$ $HLA\text{-}DR^{low}$ $CD14^+$ $CD33^+$ and suppress T cells using arginase[6]. In addition, ROS production may contribute to T cell inhibition by MDSC, and HCV-infected hepatocytes were found to promote MDSC differentiation from PBMC[7]. This might represent a mechanism of HCV-mediated immune suppression that leads to persistent infection.

ROLE OF MDSC FOR HEPATOCELLULAR CARCINOMA

Several studies have addressed the function of MDSC in liver cancer by investigating murine models of HCC. Mice bearing liver tumors show increased numbers of MDSC in liver, spleen, and bone marrow[34-37]. Remarkably, the timing of MDSC accumulation seems to be highly dependent on the tumor model studied. Mice with diethylnitrosamine (DEN) or transgenic myc-overexpression induced liver tumors, in which primary liver cancer develops slowly in the "normal" hepatic microenvironment, showed increased MDSC numbers only during late stages of the disease, while mice with orthotopic or subcutaneous tumors displayed increased MDSC numbers early on[34]. In addition, MDSC from mice with transplantable tumors showed higher suppressive capacity than MDSC from mice with DEN-induced HCC. Several studies showed that treatment with the multi-kinase inhibitor sorafenib[34,35] or an agonistic anti-CD137 antibody[37] decreased frequency of MDSC in mice bearing HCC, thereby contributing to anti-tumoral immunity.

Several soluble factors have been implicated in the recruitment of MDSC during HCC development. Tumor derived GM-CSF and KC mediated the accumulation of MDSC during hepatocarcinogenesis, and neutralization of these molecules reduced hepatic MDSC numbers[34]. Interleukin-17 (IL-17) produced by gamma/delta T cells (γδ T cells) also indirectly mediated MDSC accumulation[38]. Ma *et al*[38] showed that γδ T cell-derived IL-17 induced secretion of CXCL5 by tumor cells, which then recruited MDSC *via* engagement of CXCR2. Moreover, IL-17 also acted on the MDSC directly by enhancing their suppressive capacity and MDSC enhanced the production of IL-17 by γδ T cells through release of IL-23 and IL-1β. Similarly, γδ T cell-derived IL-17 has also been shown to recruit MDSC to the liver in HBV-transgenic mice, where they induce CD8 T cell exhaustion and HBV tolerance[5].

In DEN-induced liver carcinogenesis IL-18, is also involved in recruitment of MDSC to the liver. Li *et al*[39] demonstrated recently that TLR2-deficient mice develop more aggressive HCC than wildtype (wt) mice

associated with increased numbers of MDSC in the liver. This was mediated by IL-18 produced by hepatocytes and could be reversed through silencing of IL-18.

Interestingly, MDSC have also been associated with the development of liver metastasis. Mice with different types of intra-abdominal tumors showed a significant accumulation of MDSC in the liver that were able to potently suppress cytotoxic T cells and induce regulatory T cells[40]. Hepatic MDSC also differed from splenic MDSC in these models, expressing higher levels of immune-modulatory cytokines and being primarily of a monocytic phenotype. Similarly to HCC development, hepatic accumulation of MDSC was mediated by tumor-derived KC. This suggests that MDSC promote the development of liver metastases and may provide an explanation why human intra-abdominal cancers metastasize preferentially to the liver[41].

ROLE OF MDSC IN MOUSE MODELS OF LIVER INFLAMMATION AND FIBROSIS

The accumulation of neutrophils, monocytes and macrophages is a hallmark of acute and chronic liver inflammation. For instance, hepatic neutrophils are associated with drug-induced liver injury, alcoholic hepatitis or ischemia-reperfusion injury[42]. Hepatic macrophages are a remarkably heterogeneous population comprising myeloid cells with different origins (*e.g.*, resident Kupffer cells *vs* infiltrating monocyte-derived macrophages) and distinct properties[43]. Some of these neutrophils and macrophages have a clear immunosuppressive phenotype, prompting research on MDSC in acute and chronic liver injury.

Recently, MDSC have been studied in the context of acute liver inflammation and are usually associated with protective functions in this setting. We and others could show that MDSC accumulate in the liver during Concanavalin A (ConA)-, D-galactosamine (D-gal) and picryl chloride-induced hepatitis[3,4,44-47] and protect the liver from excessive damage. However, there seems to be controversy about which subsets are preferentially involved and which suppressive mechanisms they use. Two independent studies showed that administration of cannabidiol[4] or IL-25[3] increases the number of hepatic $CD11b^+$ $Gr1^+$ cells that ameliorated organ damage upon immune-mediated hepatitis. In this setting, the ratio of gMDSC to mMDSC was about 2:1, and T cell responses were inhibited in an arginase-dependent manner with mMDSC being more suppressive than gMDSC[4]. Consistently, we have shown that inhibiting the suppressive capacity specifically in the mMDSC subset led to severely aggravated hepatitis upon ConA-challenge[44]. Similar observations were also made by another group studying the role of FTY720, a sphingosine-1-phosphate receptor agonist, in recruitment of MDSC to the liver[46,47]. However, the suppressive function of these cells was dependent on iNOS and NO production rather than arginine depletion by Arg1. Furthermore, these studies also provided some insight into how MDSC are recruited to the liver. Similarly to what has been observed in liver cancer, MDSC accumulation was mediated *via* CXCR2[46,47]. In contrast to the aforementioned studies, Zhu *et al*[45] showed that, although both MDSC subtypes were recruited, only mMDSC were able to suppress T cell responses and limit liver damage in ConA-mediated hepatitis. This was also observed in acutely inflamed livers of $Tgf\beta1^{-/-}$ mice[48], where both subtypes of MDSC accumulated but only mMDSC were capable of suppressing T cells utilizing iNOS.

Overall, the liver provides a unique tolerogenic microenvironment, and several antigen-presenting cells contribute to the suppression of immunogenic T cell responses in the liver[49]. It has become increasingly clear that immune tolerance can also occur during chronic liver diseases. On the one hand, such tolerogenic mechanisms may limit intrahepatic immune responses and subsequent tissue injury, but on the other hand, immune tolerance may restrain eradication of pathogens and favor chronic infections[50]. Only limited data is available on the involvement of MDSC in chronic liver injury and the development of liver fibrosis. A recent study by Suh *et al*[13] indicates that bone marrow-derived MDSC can ameliorate hepatofibrogenesis through the production of IL-10, which downregulates pro-fibrotic functions of activated HSC. Interestingly, IL-10 production was induced upon contact with activated HSC *in vitro*, suggesting a mechanism for the beneficial effects observed in patients and mice with hepatic fibrosis treated with infusion of bone marrow cells[51]. On the contrary, liver fibrosis development upon chronic injury was not affected in a mouse model of transgenic overexpression of the transcription factor crem-alpha, which impairs the functionality of hepatic mMDSC[44]. Thus, more data are needed to define the possible role of MDSC in chronic inflammatory settings in the liver, and their involvement may likely vary depending on the etiology of the underlying disease, *e.g.*, autoimmunity, chronic viral hepatitis or metabolic injury.

MDSC AS THERAPEUTIC TARGETS FOR THE TREATMENT OF LIVER DISEASES

Given that MDSC are mainly associated with pathogenic functions in human chronic liver diseases such as chronic viral infections or liver cancer development, depletion of these cells and/or inhibition of their development may hold high potential in the treatment of such diseases. It has been shown that MDSC can be differentiated from murine bone-marrow cells and human PBMC *in vitro* in the presence of GM-CSF and IL-6[52-54]. Thus, these cytokines might be therapeutically targeted to avoid development of MDSC *in vivo*, but due to the various other functions of these cytokines, systemic inhibition might not be feasible and methods of local inhibition should be explored. In tumor bearing

mice depletion of MDSC using a Gr-1 specific antibody has proven to help with eradication of tumors and prevention of recurrence[55,56]. However, a more recent study reported that this antibody failed to completely eliminate hepatic MDSC[57], challenging the feasibility of this approach for liver disease therapy. Since MDSC are considered immature cells, influencing the differentiation of these cells into other myeloid cells that promote rather than inhibit immune responses could be a different therapeutic approach. Retinoic acid and vitamin D3 have both been implicated in the differentiation of MDSC to dendritic cells *in vitro* and administrations of these agents to tumor-bearing mice or cancer patients resulted in the significant improvement of anti-tumor immune responses[58-61].

In murine models of acute liver inflammation MDSC have been associated with protective rather than pathogenic functions. Therefore, it might be helpful to enhance hepatic MDSC numbers for the treatment of patients with acute inflammation or autoimmunity in the liver. The previously mentioned induction of MDSC from PBMC using GM-CSF and IL-6 would allow for the generation and expansion of autologous MDSC that can then be retransferred to the patient. The fact that adoptively transferred MDSC preferentially home to the liver[15] acts in favor of this approach allowing directed delivery of MDSC to the site of inflammation. However, migration of MDSC and "off-target" T cell suppression cannot be ruled out and should be considered in this setting.

Taken together, MDSC represent promising therapeutic targets in the treatment of liver diseases, but more extensive research is needed before these approaches can be used in clinical settings.

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EDITORIAL

Gut region-dependent alterations of nitrergic myenteric neurons after chronic alcohol consumption

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Abstract

Chronic alcohol abuse damages nearly every organ in the body. The harmful effects of ethanol on the brain, the liver and the pancreas are well documented. Although chronic alcohol consumption causes serious impairments also in the gastrointestinal tract like altered motility, mucosal damage, impaired absorption of nutrients and inflammation, the effects of chronically consumed ethanol on the enteric nervous system are less detailed. While the nitrergic myenteric neurons play an essential role in the regulation of gastrointestinal peristalsis, it was hypothesised, that these neurons are the first targets of consumed ethanol or its metabolites generated in the different gastrointestinal segments. To reinforce this hypothesis the effects of ethanol on the gastrointestinal tract was investigated in different rodent models with quantitative immunohistochemistry, *in vivo* and *in vitro* motility measurements, western blot analysis, evaluation of nitric oxide synthase enzyme activity and bio-imaging of nitric oxide synthesis. These results suggest that chronic alcohol consumption did not result significant neural loss, but primarily impaired the nitrergic pathways in gut region-dependent way leading to disturbed gastrointestinal motility. The gut segment-specific differences in the effects of chronic alcohol consumption highlight the significance the ethanol-induced neuronal microenvironment involving oxidative stress and intestinal microbiota.

Key words: Chronic ethanol consumption; Nitrergic myenteric neurons; Enteric nervous system; Nitric oxide synthase; Gut motility disorders; Intestinal microbiota

Core tip: Chronic ethanol administration causes neurodegeneration in the central nervous system. In the enteric nervous system neurodegeneration was not demonstrated, however alcohol-induced quantitative, functional and neurochemical changes of nitrergic myenteric neurons were observed in gut region-dependent way. These suggest that disturbed gastrointestinal transit characteristic to alcoholic patients due to an impairment

of a nitric oxide-mediated descending inhibition during peristalsis. The better understanding of the effects of chronic ethanol administration on enteric neurons may reveal new targets for therapy.

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INTRODUCTION

Alcoholism is one of the world's leading risk factor for morbidity, disability and mortality. In 2012, 5.9% of all global deaths in the world were attributable to alcohol abuse. Chronic alcohol consumption is a component cause of more than 200 disease and injury conditions[1].

The majority of absorbed ethanol is metabolized in the liver, but disassembly of ethanol also occurs in the whole length of the gastrointestinal (GI) tract, including the oral cavity, the esophagus, the stomach, and the small and large intestines. Ethanol is metabolized oxidatively into acetaldehyde by alcohol dehydrogenase, by the microsomal ethanol oxidizing system cytochrome P4502E1, and by catalase in the peroxisomes[2,3].

Ethanol and its oxidative and non-oxidative metabolites can be found throughout the GI tract, where they can interfere with several functions, including the intestinal barrier[2], GI motility and absorption of nutrients[4-6].

Animal models are available to investigate alcohol-related diseases[7-20], however there is still a need for animal models resembling more the human condition. It is well documented that alcohol ingestion results neuroinflammation and neurodegeneration in humans and animals[19]. In the induction of neuronal apoptosis, oxidative stress plays an important role[21]. Toxic and metabolic effects of ethanol vary in brain regions, the most affected regions are the frontal lobes, the cortical limbic-circuits and the cerebellum. Skeletal muscle, and peripheral nerves are also important targets of chronic alcohol-related metabolic injury and degeneration[22,23].

In this review the effects of chronic ethanol consumption on the enteric nervous system (ENS) are highlighted, particular the changes in the quantitative properties of nitrergic myenteric neurons and related motility disturbances in the different parts of the GI tract.

ALCOHOL AND NOS CONTAINING NEURONS

In the ENS, nitric oxide (NO) plays a critical role in mediating non-adrenergic, non-cholinergic relaxation of the intestinal smooth muscle in a gut regionally different way[24-26]. High concentrations of ethanol are reached only in the duodenum and jejunum[5,27,28], however the concentration of ethanol reached in the ileum is not significantly different to the levels in the blood[27]. Therefore, the neuronal NO may be altered directly by the ethanol in the duodenum, while by the different oxidative and reductive metabolites in the different intestinal segments after chronic ethanol consumption. More findings provide evidence that effects of ethanol on NO system of intestinal relaxation[6,29] is responsible to the impaired motor function leading gut motility disorders[5,30,31]. NO is synthesized by the neuronal (n), endothelial (e) and inducible (i) nitric oxide synthases (NOSs)[32], and now, numerous investigations have already confirmed that all the NOSs are constitutively expressed in the myenteric neurons[33,34]. However, the effects of chronic alcohol intake on the density of nitrergic myenteric neurons, the amount of the three NOSs and/or their activity in different parts of the GI tract have been poorly investigated.

Therefore, in the last ten years we concentrated our research on the alcohol-induced alterations of nitrergic myenteric neurons in different gut regions[6,31,35,36]. We established a model suitable to study the NOS activities, protein content and the number of total and nNOS-immunoreactive myenteric neurons (Figure 1) in the duodenum, jejunum, ileum and colon of control and ethanol-exposed animals[35]. The activity of constitutive NOS (cNOS, both neuronal and endothelial) was 20 times higher in the proximal colon than in any part of the small intestine in control animals. Except of duodenum cNOS activity decreased significantly after chronic ethanol consumption. Under physiological conditions, the iNOS activity was also higher in the distal gut segments, but it did not change by the effects of ethanol. Similar results were observed in NOS protein content of tissue samples; the nNOS density of colonic fractions was more than twice as high as those of the samples prepared from the other gut segments and it also decreased after chronic ethanol consumption. The densities of eNOS-fractions were very weak and differences were not revealed in different intestinal samples and conditions. In intestinal whole-mount preparations from control rats, the number of nNOS-immunoreactive neurons was the highest in the colon. After ethanol exposure the decrease in the nitrergic cell number was significant in the whole length of the gut (Figure 2), however the greatest decrease in density of nitrergic neurons was observed in the colon[35]. The total number of myenteric neurons labelled with HuC/HuD pan-neuronal marker did not differ between controls and ethanol-drinking rats which suggest that chronic alcohol administration did not result in significant cell loss, but primarily impaired the nitrergic pathways in regionally different way.

Reduced number of nNOS-expressing neurons after chronic alcohol intake was also demonstrated in the murine jejunum[31] without changing in the total neuronal number. Both results indicate that chronic alcohol consumption leads to reduced nNOS expression

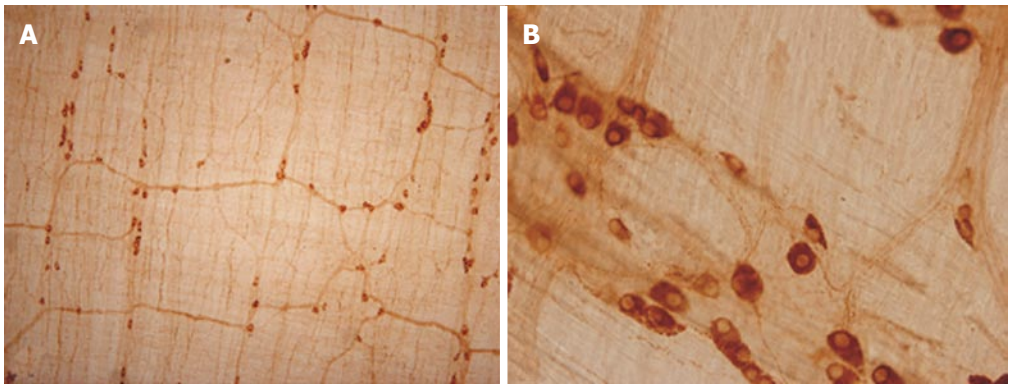

Figure 1 Representative light micrographs of whole mount preparation on the myenteric neurons of rat ileum after neuronal nitric oxide synthase immunohistochemistry. A: × 100; B: × 400.

resulting in motility disorders. In another study[6], the bio-imaging of basal NO synthesis of individual myenteric neurons was validated by loading the whole-mount preparations with the fluorescent indicator DAF-FM[37,38]. Based on DAF-FM recordings, chronic alcohol consumption induced a markedly increased basal NO synthesis in myenteric neurons as well as in glial cells or smooth muscle cells, indicates that chronic alcohol intake caused a general overproduction of NO in the jejunal gut wall. They confirmed reduced proportion of nNOS-expressing myenteric neurons and an increase of the proportion of iNOS-immunoreactive neurons was also revealed in murine small intestine[6]. Interestingly, the percentage of iNOS-containing neurons is in reasonable agreement with the measured percentages of neurons that produced NO but were not immunoreactive for nNOS. Others has also demonstrated that ethanol increased the amount of intestinal iNOS[36,39] and content of NO[39] in rats. Ethanol-induced NO overproduction appears to be relevant to the intestinal barrier dysfunction and alcoholic gut leakiness[40].

Besides myenteric neurons, the presence of the three NOSs shows characteristic cell type-specific distribution in enteric smooth muscle cells and capillary endothelium[34]. In accord with recent studies[41,42], we hypothesized that the presence of the three NOSs with similar functions in the same type of cells, the gut wall is able to adapt to different pathological conditions. To evaluate the possible rearrangement of the cellular and subcellular NOS compartments in response to chronic ethanol treatment post-embedding immuno-electron microscopy was used in different gut segments and cell types. Counting gold particles labelling different NOSs, the nNOS labels were in general the most numerous under normal conditions[36] which is in agreement with the finding of an earlier study[43] and strengthen that in the GI tract, nNOS is the main source of NO. However in the different intestinal segments and cellular compartments, well-pronounced differences were observed in the number of nNOS labels under physiological and alcoholic conditions. After chronic ethanol consumption, the numbers of nNOS labels are decreased in one intestinal segment and increased in another suggest significant differences in the microenvironment in different gut regions. Interestingly, the quantitative features of eNOS labels were changed in the opposite way to those in nNOS signing after ethanol intake. For example, while the number of nNOS gold particles decreased by more than 50% in the ganglia of duodenum, the eNOS labels approximately doubled here[36]. Depending on the investigated gut segment and type of NOS, a pronounced subcellular realignment of NOS labels was also found in ethanol-treated rats. The opposite alterations of eNOS and nNOS and subcellular rearrangement of NOS compartments may reflect a functional plasticity, in which different NOSs can replace each other to help maintenance the optimum NO level even under pathological condition.

CHRONIC ETHANOL ADMINISTRATION AFFECTS GASTROINTESTINAL MOTILITY

Although the effects of ethanol consumption on gastrointestinal motility is well documented[44-49], even the opposite effects of acute and chronic administration of alcohol on GI transit have demonstrated, the mechanisms underlying impaired smooth muscle contractility are poorly understood and several conflicting data are present.

To reinforce the pathogenic role of NO in the ENS during chronic alcohol treatment, we investigated possible changes in the proportion of nitrergic myenteric neurons in relation to GI motility disturbances observed after chronic alcohol consumption in a murine model[31].

We demonstrated that chronic alcohol consumption affects gastric emptying and small intestinal transit *in vivo* (Figure 3). Migration of an Evans blue bolus throughout the stomach and small intestine was significantly delayed in chronic alcohol-treated mice when compared with controls receiving tap water. These findings point to an effect of chronic alcohol treatment on both stomach and small intestinal motility.

To elucidate whether this delay in intestinal transit could be associated with altered nitrergic relaxation of smooth muscle, we performed *in vitro* organ bath

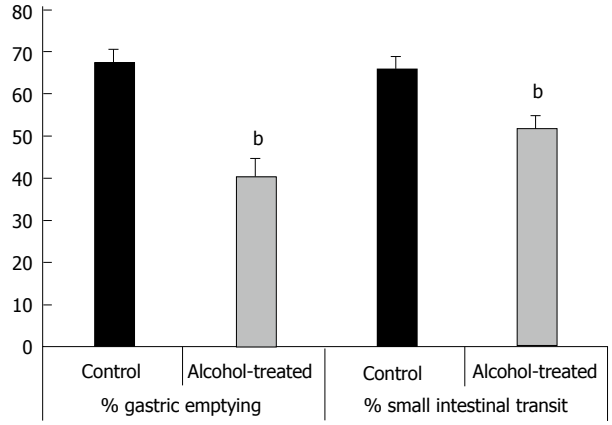

Figure 2 ***In vivo*** **measurement of % gastric emptying and % small intestinal transit in control and alcohol-treated mice (Bagyánszki *et al*[6] 2010).** $^{b}P <$ 0.0001 *vs* control.

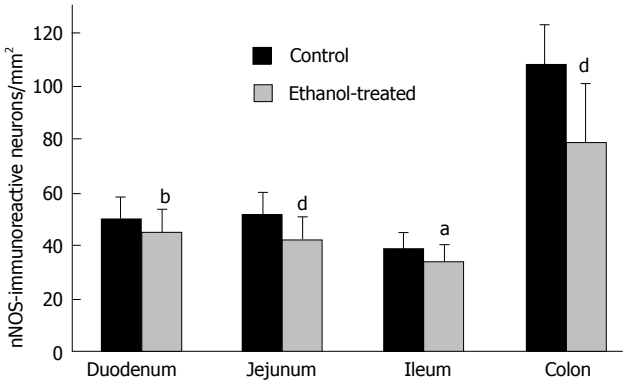

Figure 3 Gut-region-specific changes in the density of nNOS-immunoreactive myenteric neurons after chronic ethanol consumption (Krecsmarik *et al*[35] 2006). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control.

experiments. Jejunal muscle strips relaxed to electrical field stimulation (EFS) and these relaxations were mimicked by exogenous NO. Relaxations to EFS were blocked by the NOS inhibitor L-NNA, confirming that they are mediated by NO[50]. In chronic alcohol-treated mice, the nitrergic relaxations to EFS were significantly decreased, whereas those to increasing concentrations of exogenous NO did not differ between chronic alcohol-treated and control mice. This finding indicates that the effect exerted by chronic alcohol consumption on smooth muscle relaxation is not because of a defective responsiveness of the smooth muscle to NO but appears to originate from impaired nitrergic neuronal activity[31].

Recently Yazir *et al*[51] found that chronic alcohol consumption impairs relaxant and contractile responses of both esophageal tunica muscularis mucosae and lower esophageal sphincter smooth muscle. Similarly to our results they found decreased nNOS immunoreactivity in esophageal myenteric plexus in alcohol-exposed group compared to control groups[51].

ALCOHOL AND GUT MICROBIOTA

It is well documented that the anatomical, functional, and pathological regionality of the gastrointestinal tract develops under strict genetic control[52,53], which itself result in the unique susceptibility of the neurons to pathological conditions in different intestinal segments. The gut region-specific neuronal damage demonstrated in rats with chronic ethanol consumption[6,31,35,36] indicates the importance of the molecular differences in the microenvironment of nitrergic neurons located in different gut segments[36]. It has recently evidenced that after chronic ethanol consumption, the three NOS isoforms were affected differentially not only in the myenteric neurons but also in mesenteric capillaries running in the vicinity of myenteric ganglia and smooth muscle cells[36]. Among the many factors that are implicated in this regionally distinct pathologic microenvironment of neurons in different gut segments, the intestinal microbiota got recently in the focus of research gastrointestinal diseases[54,55].

The composition of gut microbiome and the amount of bacteria is also unique along the oro-anal gut axis. The upper gastrointestinal tract does not harbour a rich of microbial concentration due to gastric acid, biliary and pancreatic secretion, while in the colon the highest density of bacterial community is found[56,57] with potential role in prevention, several metabolic activities and gut permeability[58-60]. Alcohol has been shown to increase in total number of bacteria in jejunum[61] and also result in duodenal bacterial overgrowth[62,63]. Others[64] also demonstrated that chronic ethanol feeding caused an increase in the abundance of the gram negative *Proteobacteria* including several pathogenic species and gram positive *Actinobacteria*, as well as resulted in a decline of *Bacteroidetes* and *Firmicutes* phyla. The balance in the composition of microbiome is critical to maintain gut homeostasis, therefore the breakdown of it associated with endotoxemia, lipopolysaccharides translocation and several immunological reactions[65]. Elevation of the growth of gram negative bacteria results in augmentation of lipopolysaccharides like endotoxins, a component of gram negative bacterial wall. Endotoxins release several pro-inflammatory cytokines from activated macrophages[66,67] which lead the alteration of intestinal barrier through disruption of tight junctions[68] and contribute to the progression of alcoholic liver disease, cirrhosis or alcoholic pancreatitis[55,69]. Increased gut permeability allows to endotoxins passing into the bloodstream creating harmful cycles[70]. However, Zhong *et al*[71] found that the gut leakiness after chronic alcohol exposure occurs in the ileum but not in the duodenum or jejunum. They also observed that alcohol exposure caused ROS accumulation in the small intestine with strongest labelling in the ileum. In parallel with oxidative stress, the zinc dyshomeostasis was also found gut region-specific as a consequence of ethanol exposure; the zinc status (an important trace element of all the major cell functions) was not affected in the duodenum

and jejunum but significantly decreased in the ileum[71]. Besides oxidative stress, alcohol reactive metabolites also have been suggested to critically mediate alcohol-induced intestinal barrier dysfunction[72,73]. Acetaldehyde is produced in a high concentration through ethanol metabolism by bacterial alcohol dehydrogenase[74,75] mediated mainly by aerobic or facultative anaerobic bacteria in the colon[76-78]. Based on these findings, further investigations on the region-specific composition of gut microbiome and alcohol-related alterations of intestinal microbiota in different gut segments should be performed to reveal the underlying events.

DISCUSSION

Endogenous NO is largely involved in the regulation of gut motility, secretion and blood flow[79-81]. More findings provide evidence that nitrergic subpopulation of the myenteric neurons is especially susceptible to different pathological conditions[35,36,81-85]. Furthermore, the NOS neurons are more sensitive to damage than other enteric neurons[86]. Among the possible reasons involving NOS neurons in enteric neuropathies, intracellular Ca^+ concentration is thought to be critical[87]. In stress, neurons cannot maintain the optimal intracellular Ca^+ level. Elevated Ca^+ activates NOSs result in excessive production of free radicals as NO or peroxynitrite which lead cytotoxicity of neurons[86,87]. It has also been demonstrated that impairment of nitrergic myenteric neurons after chronic ethanol consumption is strictly gut region-dependent[35,36], which emphasize the importance of neuronal microenvironment. Therefore, to reveal the region-specific structural and molecular differences along the whole length of GI tract is essential to outline new directions in the diagnosis and the therapies GI diseases in chronic alcoholism.

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EDITORIAL

Can high resolution manometry parameters for achalasia be obtained by conventional manometry?

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Abstract

High resolution manometry (HRM) is a new technology that made important contributions to the field of gastrointestinal physiology. HRM showed clear advantages over conventional manometry and it allowed the creation of different manometric parameters. On the other side, conventional manometry is still wild available. It must be better studied if the new technology made possible the creation and study of these parameters or if they were always there but the colorful intuitive panoramic view of the peristalsis from the pharynx to the stomach HRM allowed the human eyes to distinguish subtle parameters unknown or uncomprehend so far and if HRM parameters can be reliably obtained by conventional manometry and data from conventional manometry still can be accepted in achalasia studies. Conventional manometry relied solely on the residual pressure to evaluate lower esophageal sphincter (LES) relaxation while HRM can obtain the Integrated Relaxation Pressure. Esophageal body HRM parameters defines achalasia subtypes, the Chicago classification, based on esophageal pressurization after swallows. The characterization of each subtype is very intuitive by HRM but also easy by conventional manometry since only wave amplitudes need to be measured. In conclusion, conventional manometry is still valuable to classify achalasia according to the Chicago classification. HRM permits a better study of the LES.

Key words: Achalasia; Esophagus; High resolution manometry; Conventional manometry; Lower esophageal sphincter; Esophageal body; Chicago classification

Core tip: High resolution manometry is a new technology with clear advantages over conventional manometry. It is unclear; however, if new parameters created after this technology can be obtained by

conventional manometry especially in achalasia cases. We found that conventional manometry is still valuable to classify achalasia according to the Chicago classification but high resolution manometry permits a better study of the lower esophageal sphincter.

Herbella FAM, Patti MG. Can high resolution manometry parameters for achalasia be obtained by conventional manometry? *World J Gastrointest Pathophysiol* 2015; 6(3): 58-61 Available from: URL: http://www.wjgnet.com/2150-5330/full/v6/i3/58.htm DOI: http://dx.doi.org/10.4291/wjgp.v6.i3.58

High resolution manometry (HRM) made important contributions to the field of gastrointestinal physiology. HRM, in comparison to conventional manometry, not only brings more comfort and speediness to the test[1], a more intuitive comprehension of the plots compared to tracings[1] even for beginners[2], and a lesser degree of interobserver and intraobserver interpretation variability[3] but also HRM proved to be advantageous in the following parameters: (1) evaluation of gastric motility[4]; (2) the correct evaluation of the lower esophageal sphincter (LES) relaxation and esophagogastric junction flow avoiding movement artifacts and correlating it temporally with swallowing[5]; (3) the identification of segmental defects of peristalsis not covered by the spacing of sensors in conventional systems[6]; and (4) the motility and temporal correlation of the pharyngo-upper esophageal complex due to the rapid response and circunferentiality of the solid-state sensors, and compensation for movement artifacts[7].

The detailed view provided by HRM permitted the creation of new manometric parameters and a new classification of motility disorders, the Chicago classification[8], recently simplified and reviewed in his 3.0 version[9] with a 4.0 version under creation to incorporate intraluminal impedance as well. The Chicago classification made 3 major contributions: (1) defined an objective parameter to measure LES relaxation, the integrated relaxation pressure (IRP); (2) classified achalasia in 3 distinct subtypes; and (3) showed a prognostic value of manometry parameters[10].

It is still elusive; however, if the new technology made possible the creation and study of these parameters or if they were always there but the colorful intuitive panoramic view of the peristalsis from the pharynx to the stomach HRM allowed the human eyes to distinguish subtle parameters unknown or uncomprehend so far (see examples on Figure 1). This lead to questioning if HRM parameters can be obtained by conventional manometry and data from conventional manometry still can be accepted in achalasia studies since HRM is not wildly available due to the high cost of the system and catheters.

Achalasia is a rare primary esophageal motor disorder characterized by aperistalsis and absent or incomplete relaxation of the LES[13]. Dysphagia and regurgitation are common symptoms of the disease that

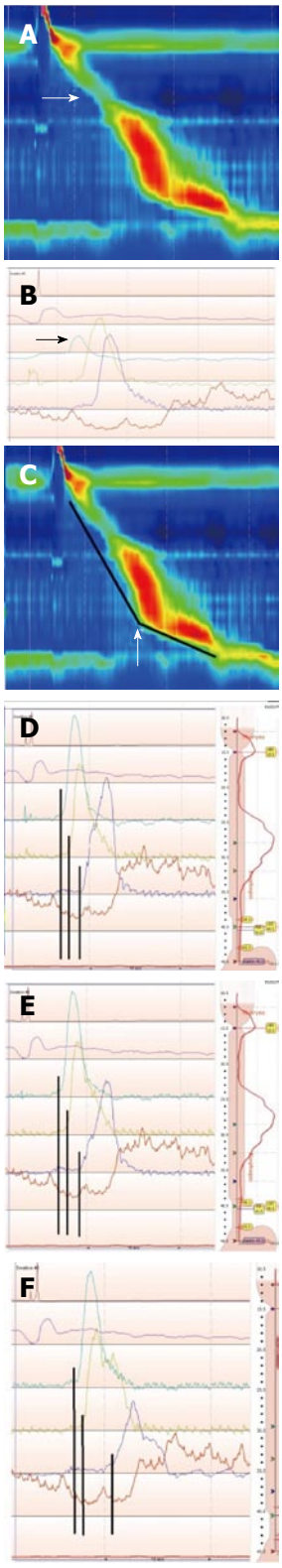

Figure 1 Example of high resolution parameters identifiable at the conventional manometry. A: The peristaltic gap at transition zone (change from striated to smooth muscle in the proximal esophagus - arrow) has been fully explored with high resolution manometry[11] but it was well-known and identifiable as low amplitude waves at the proximal esophagus (arrow), although the clinical significance not comprehended, by conventional manometrists (B)[12]; C: The contractile deceleration point (CDP) represents the inflexion point in the contractile front propagation velocity in the distal esophagus representing the motility of the ampulla (arrow). Conventional manometry neglected time and privileged only amplitudes. A progressive latter onset of the distal wave (CDP) can be noticed from 3 to 1 cm above the lower esophageal sphincter upper border (D-F).

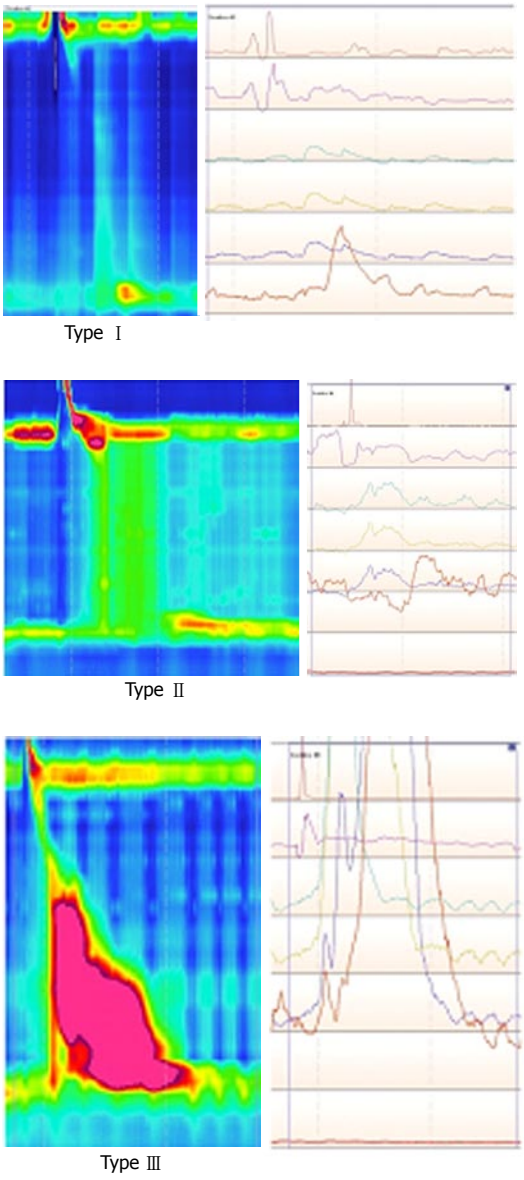

Figure 2 Chicago classification subtypes at the light of high resolution manometry (left) and conventional manometry (right). Type Ⅰ (no distal pressurization), type Ⅱ (panesophageal pressurization), and type Ⅲ (premature spastic contractions).

in association with a dilated esophagus on the barium esophagram makes the diagnoses of this disease not difficult[14]. Esophageal manometry; however, is useful not only for the diagnosis in difficult cases, especially without esophageal dilatation, but it seems to predict therapeutic outcomes[10], usually accomplished *via* endoscopic forced dilatation of the cardia or surgical Heller's myotomy and fundoplication[15].

Conventional manometry relied solely on the residual pressure to evaluate LES relaxation[16]. IRP (Integrated Relaxation Pressure - the average minimum esophagogastric junction pressure for 4 s of relaxation within 10 s of swallowing) is virtually impossible to be calculated in conventional tracings.

Esophageal body HRM parameters define achalasia subtypes, the Chicago classification, based on esophageal pressurization after swallows. The characterization of each subtype is very intuitive by HRM but also easy by conventional manometry since only wave amplitudes need to be measured (Figure 2). In fact, some well-known papers successfully applied the classification in patients submitted to conventional manometry[17,18]. Moreover, type III corresponds to the old terminology "vigorous achalasia"[8].

Different studies showed that achalasia type II shows the best response and type Ⅲ the worst response to either Heller's myotomy or endoscopic pneumatic dilatation[19]. The prognostic value of manometric parameters to define therapy outcomes is; however, not new to HRM. Long before Chicago classification, some Brazilians surgeons noticed poorer results for patients with lower amplitudes of the simultaneous waves observed at the conventional manometry (< 20 mmHg) precluding the choice of a Heller myotomy opting for an esophagectomy in these cases[20]. Very interestingly too, some authors found prognostic value for the basal pressure of the LES at conventional manometry[21], but this was never tested for HRM.

In conclusion, conventional manometry is still valuable to classify achalasia according to the Chicago classification. HRM permits a better study of the LES.

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MINIREVIEWS

Iron deficiency anemia in inflammatory bowel disease

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Abstract

Anemia is a common extraintestinal manifestation of inflammatory bowel disease (IBD) and is frequently overlooked as a complication. Patients with IBD are commonly found to have iron deficiency anemia (IDA) secondary to chronic blood loss, and impaired iron absorption due to tissue inflammation. Patients with iron deficiency may not always manifest with signs and symptoms; so, hemoglobin levels in patients with IBD must be regularly monitored for earlier detection of anemia. IDA in IBD is associated with poor quality of life, necessitating prompt diagnosis and appropriate treatment. IDA is often associated with inflammation in patients with IBD. Thus, commonly used laboratory parameters are inadequate to diagnose IDA, and newer iron indices, such as reticulocyte hemoglobin content or percentage of hypochromic red cells or zinc protoporphyrin, are required to differentiate IDA from anemia of chronic disease. Oral iron preparations are available and are used in patients with mild disease activity. These preparations are inexpensive and convenient, but can produce gastrointestinal side effects, such as abdominal pain and diarrhea, that limit their use and patient compliance. These preparations are partly absorbed due to inflammation. Non-absorbed iron can be toxic and worsen IBD disease activity. Although cost-effective intravenous iron formulations are widely available and have improved safety profiles, physicians are reluctant to use them. We present a review of the pathophysiologic mechanisms of IDA in IBD, improved diagnostic and therapeutic strategies, efficacy, and safety of iron replacement in IBD.

Key words: Iron deficiency anemia; Inflammatory bowel disease; Hepcidin; Ferritin; Oral iron; Intravenous iron

Core tip: Iron deficiency anemia (IDA) is increasingly recognized as a common complication in patients with inflammatory bowel disease (IBD). IDA has a significant impact on quality of life and health care costs. This comprehensive review article discusses the latest advances in understanding the pathophysiologic mechanisms involved in development of IDA in patients with IBD, and reviews new diagnostic tests and therapeutic options with high safety indexes for the management of IDA. This article aims at increasing physician awareness and understanding of the complex mechanisms involved in IDA, and the current cutting-edge approach for the management of IDA in patients with IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) describes a set of chronic gastrointestinal illnesses, including Crohn's disease (CD) and ulcerative colitis (UC), of multifactorial etiology, which proceed with periods of relapse and remission. Extraintestinal complications are common in IBD, and are reported in more than 25% of patients[1]. Anemia is one of the most common manifestations of IBD[2]. One-third of patients with IBD have hemoglobin levels below 12 g/dL[3]. The anemic state is strongly correlated with quality of life, and is an important problem in the therapeutic management of patients with chronic disease[4].

ABSOLUTE AND FUNCTIONAL IRON DEFICIENCY

Most cases of anemia in patients with IBD result from functional or absolute iron deficiency. Functional iron deficiency is a state in which there is insufficient availability of iron for incorporation into erythroid precursors despite normal or increased body iron stores[5,6]. In patients with absolute iron deficiency, iron is stored in the bone marrow. Other parts of the monocyte-macrophage system in the liver and spleen become depleted, making iron unavailable for normal or increased rates of erythropoiesis. This may occur as the result of poor dietary intake of iron, reduced iron absorption, and/or increased blood loss.

SIGNS AND SYMPTOMS OF IRON DEFICIENCY ANEMIA

Signs and symptoms of iron deficiency depend on the severity and chronicity of the anemia, in addition to the usual signs of anemia, including fatigue, pallor, and reduced exercise capacity. Cheilosis and koilonychia are signs of advanced tissue iron deficiency which are not frequently seen in the modern world, due to early diagnosis and timely correction.

Key symptoms of anemia, such as dyspnea and tachycardia, are caused by decreased blood oxygen levels and peripheral hypoxia. Compensatory blood shifting from the mesenteric arteries may worsen perfusion of the intestinal mucosa[7]. Motility disorder, nausea, anorexia, and even malabsorption have been attributed to anemia. Reduced metabolic and energy efficiency during physical activity also contribute to weight loss in anemia[8].

Central hypoxia may lead to symptoms such as headache, dizziness, vertigo, or tinnitus. Several studies have confirmed that treatment of anemia improves cognitive function[9]. Iron is a component of hemoglobin myoglobin, cytochromes, and many other enzymes. Thus, anemia negatively impacts almost every aspect of daily life in patients with IBD. Men with iron deficiency anemia (IDA) may suffer from impotence. Loss of libido contributes to impaired quality of life in both sexes[10]. In addition, latent iron deficiency may be responsible for "non-hematological" symptoms such as hair loss, paresthesias of the hands and feet, and reduced cognitive function, and may also be significantly associated with restless leg syndrome.

CLINICAL RELEVANCE

Anemia and iron deficiency have a dramatic impact on patients' quality of life, yet anemia in patients with IBD is still underdiagnosed and undertreated. In the United States, annual emergency room visits for anemia average around 209000[11], drastically maximizing health care costs. In a patient population with the predisposition for anemia, like patients with IBD, early diagnosis and management of iron deficiency can promptly reduce hospital visits, improve quality of life, reduce loss of work, and, ultimately, lower health care costs.

CAUSES OF IRON DEFICIENCY ANEMIA IN IBD

Anemia in IBD patients involves multiple pathogenic mechanisms resulting in low hemoglobin levels and compromised quality of life. Although ongoing blood loss from chronically inflamed intestinal mucosa and micronutrient deficiency (iron and B12) are the main mechanisms underlying the development of anemia in patients with IBD, chronic inflammation, hemolysis, and medication-induced myelosuppression may also play important roles in both the development of anemia and the management of this condition[12,13]. Anemia of chronic disease (ACD) and IDA are the two most common causes of anemia in patients with IBD[14,15].

Table 1 Causes of anemia in inflammatory bowel disease

Common causes	Less common causes	Rare causes
Iron deficiency anemia	B12 deficiency	Hemolysis
Anemia of chronic inflammation	Folic acid deficiency	Chronic renal insufficiency
	Medications causing B12/Folate deficiency	Myelodysplastic syndrome
		Medication induced aplasia
		Congenital hemoglobinopathies
		Protein starvation
		Anemia in liver disease

Patients with IDA and concomitant ACD tend to have more severe anemia compared with patients with ACD alone[14]. Table 1 presents the causes of anemia in patients with IBD. Figure 1 outlines causes of IDA.

PATHOPHYSIOLOGY OF IRON DEFICIENCY ANEMIA IN IBD

Up to 3-4 g of iron is stored in the human body. Around 1-2 mg of iron is lost every day through desquamation of epithelial cells of the skin, gastrointestinal tract, bile ducts, and urinary tract, and through blood loss in menstruating women[16].

Iron homeostasis is strictly maintained by iron absorption from the duodenal enterocytes, and is tightly regulated by hepcidin (Figure 2). Hepcidin is a 25-amino-acid peptide hormone, has intrinsic antimicrobial activity, and is an acute-phase protein that is primarily synthesized by hepatocytes[17]. Cellular targets for hepcidin and iron-exporting cells are villous enterocytes, reticuloendothelial macrophages, and hepatocytes. Hepcidin binds to the basolateral transporter and iron exporter ferroportin 1, leading to its phosphorylation, internalization by binding to JAK 2, and lysosomal degradation, thus preventing iron release into the plasma[16]. Increased hepcidin levels downregulate ferroportin, thereby reducing iron efflux from the enterocytes and macrophages, causing hypoferremia. The increase in enterocyte iron content reduces the expression of enterocyte brush border reductase (Dcytb) and divalent metal transporter 1 (DMT1) on villous enterocytes, inhibiting dietary iron absorption causing iron deficiency anemia[18]. Therefore, by regulating the expression of DMT1 and ferroportin, hepcidin acts as a negative regulator of iron absorption in the duodenum and of iron release from the enterocytes and macrophages.

Hepcidin expression is upregulated by iron overload, infection, and inflammation, through proinflammatory cytokines such as interleukin (IL)-6 *via* the JAK 2 mediated STAT 3 signaling, thus limiting iron absorption[16,19]. Hepcidin expression is downregulated by hypoxia, oxidative stress, iron deficiency anemia, and ineffective erythropoiesis, thus increasing iron availability (Figure 2)[20].

Hepcidin expression is mediated by two signaling pathways that involve bone morphogenetic protein (BMP) and transferrin receptor 2 (TfR2; Figure 3). The BMP signaling pathway includes BMP 6, hemojuvelin (HJV), and SMAD4, which constitute the major signaling pathway for hepcidin expression; TfR2 and the hereditary hemochromatosis protein (HFE)-dependent signaling pathway modulate this response[21,22]. In high iron conditions, transferrin-iron in the plasma forms a complex with HFE and TfR2 to promote hepcidin expression[21]. In iron-deficient conditions, there is downregulation of TfR2 and upregulation of TfR1. HFE is sequestered by TfR1, preventing its interaction with TfR2, thereby downregulating hepcidin expression (Figure 3)[21,23].

DIAGNOSTIC WORK-UP OF IRON DEFICIENCY ANEMIA IN IBD

Healthcare providers screen for IDA by measuring hemoglobin, serum ferritin, and C-reactive protein (CRP). Based on expert opinion and common clinical practice, screening is recommended at least every 3 mo for outpatients with active disease, and once every 6 to 12 mo for patients in remission or with mild disease; screening is not applicable to hospitalized patients[24].

The World Health Organization (WHO) definitions of anemia also apply to patients with IBD. WHO defines anemia as hemoglobin levels < 13 g/dL (hematocrit < 39%) in males, < 12 g/dL (hematocrit < 36%) in nonpregnant females, and < 11 g/dL (hematocrit < 33%) in pregnant females[25]. Severe IDA is defined as hemoglobin levels < 10 g/dL.

If a patient meets WHO criteria for anemia, a basic anemia workup should be initiated to determine the cause of anemia. The basic workup includes serum ferritin, transferrin, transferrin saturation, and CRP levels. If the cause of anemia is unclear despite the results of the above workup, more extensive testing is recommended. Further tests include vitamin B12, folic acid, haptoglobin, lactate dehydrogenase, creatinine, and reticulocyte counts[24].

Both IDA and ACD often coexist with IBD, and the treatment for each differs. There is no single laboratory parameter that differentiates one from the other. Consequently, supplementary laboratory tests are required to differentiate IDA from ACD. These tests include soluble transferrin receptor, soluble transferrin receptor-ferritin index, reticulocyte hemoglobin concentration, zinc protoporphyrin, the percentage of hypochromic red cells, and hepcidin levels[16]. IDA in IBD is diagnosed based on a combination of factors, taking inflammation into account. The laboratory findings in IDA, ACD, mixed IDA, and ACD are shown in Table 2[16,22].

Serum ferritin is a measure of stored iron content in the reticuloendothelial system; in absolute iron

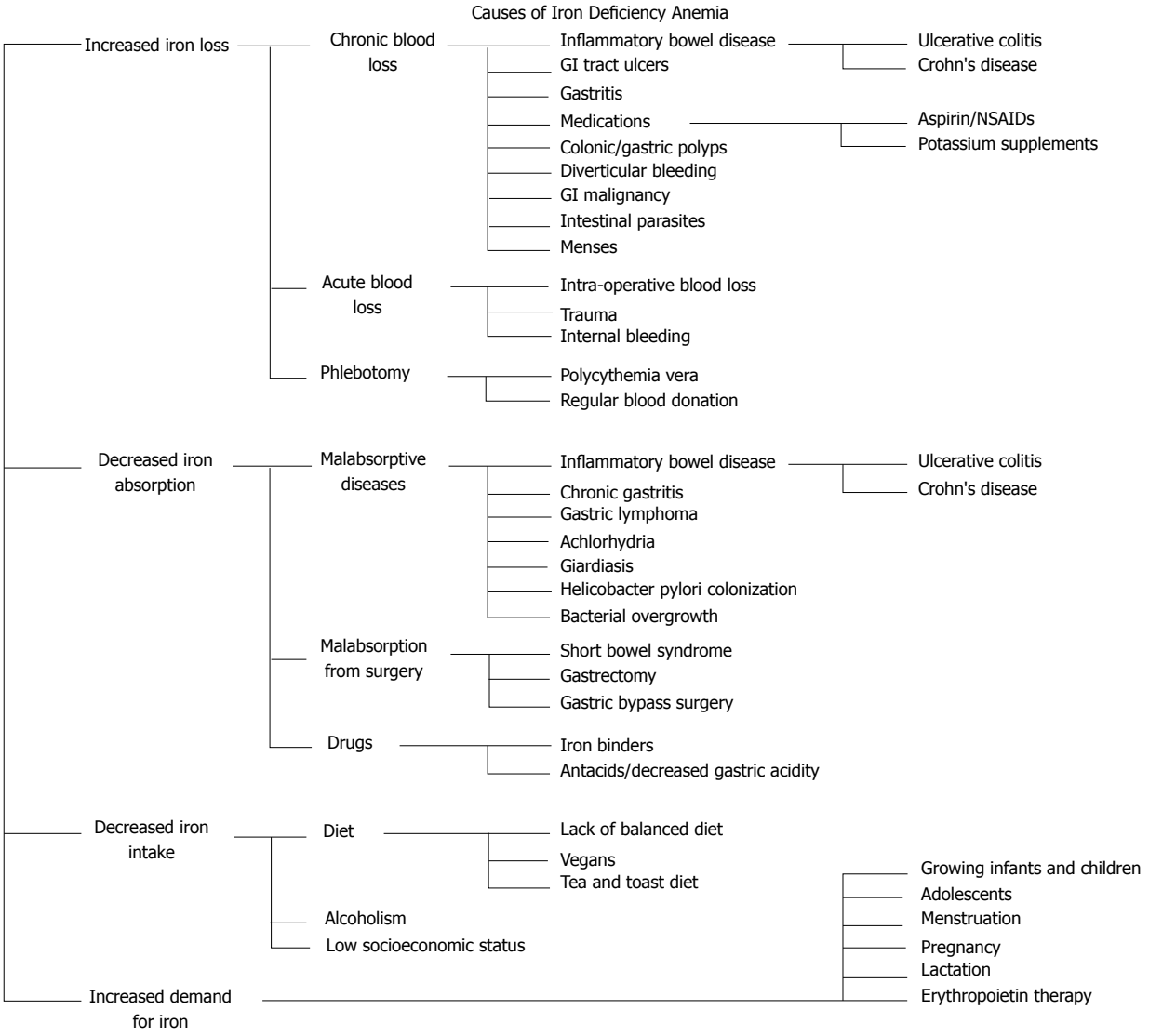

Figure 1 Causes of iron deficiency anemia. GI: Gastrointestinal; NSAIDs: Nonsteroidal anti-inflammatory drugs.

Table 2 Laboratory findings in iron deficiency anemia, anemia of chronic disease, mixed iron deficiency anem and anemia of chronic disease[16,31]

Laboratory measures	IDA	ACD	IDA and ACD
Serum iron	↓	↓	↓
Hemoglobin	↓	↓	↓
MCV	↓	↓ or normal	↓ or normal
CRP	Normal	↑↑	↑
Serum ferritin	↓	↑	↑ or normal
Transferrin	↑	↓ or normal	↓
Transferrin saturation	↓	↓	↓
sTfR	↑	↓	↑ or normal
sTfR- Ferritin index	High (> 2)	Low (< 1)	High (> 2)
Reticulocyte Hb content (CHr, pg)	< 28	⩾ 28	< 28
Zinc protoporphyrin (μmol/mol heme)	> 40	< 40	> 40
Percentage of hypochromic RBC	> 5	< 5	> 5
Hepcidin	↓	↑	↑

↓: Low/decreases; ↑: High/increases; ↑↑: Very high; IDA: Iron deficiency anemia; ACD: Anemia of chronic disease; MCV: Mean corpuscular volume; CRP: C-reactive protein; sTfR: Soluble transferrin receptor; Hb: Hemoglobin; RBC: Red blood cell.

deficiency, the serum ferritin concentration is < 15 μg/L[16]. Serum ferritin is an acute-phase reactant; normal or high levels may be found in inflammatory conditions despite iron deficiency[26]. Therefore, the guidelines recommend that in patients with quiescent IBD without biochemical or clinical evidence of inflammation, iron deficiency is defined as serum ferritin < 30 g/L or transferrin saturation (TSAT) < 16%[24]. In the presence of active IBD with inflammation, as evidenced by elevated CRP, the guidelines give a cut-off level of serum ferritin < 100 g/L to increase sensitivity and specificity[24,27].

Microcytosis (low mean corpuscular volume, MCV) and hypochromia (low mean corpuscular hemoglobin, MCH), available from the complete blood count, are indicators of absolute iron deficiency. High MCV is found secondary to vitamin B12 and folate deficiency, the use of certain medications (thiopurines; azathioprine or 6-mercaptopurine), alcoholism, and hypothyroidism[24]. Therefore, normal or high MCV does not exclude IDA as a possibility. In patients with ACD, MCV may be low or

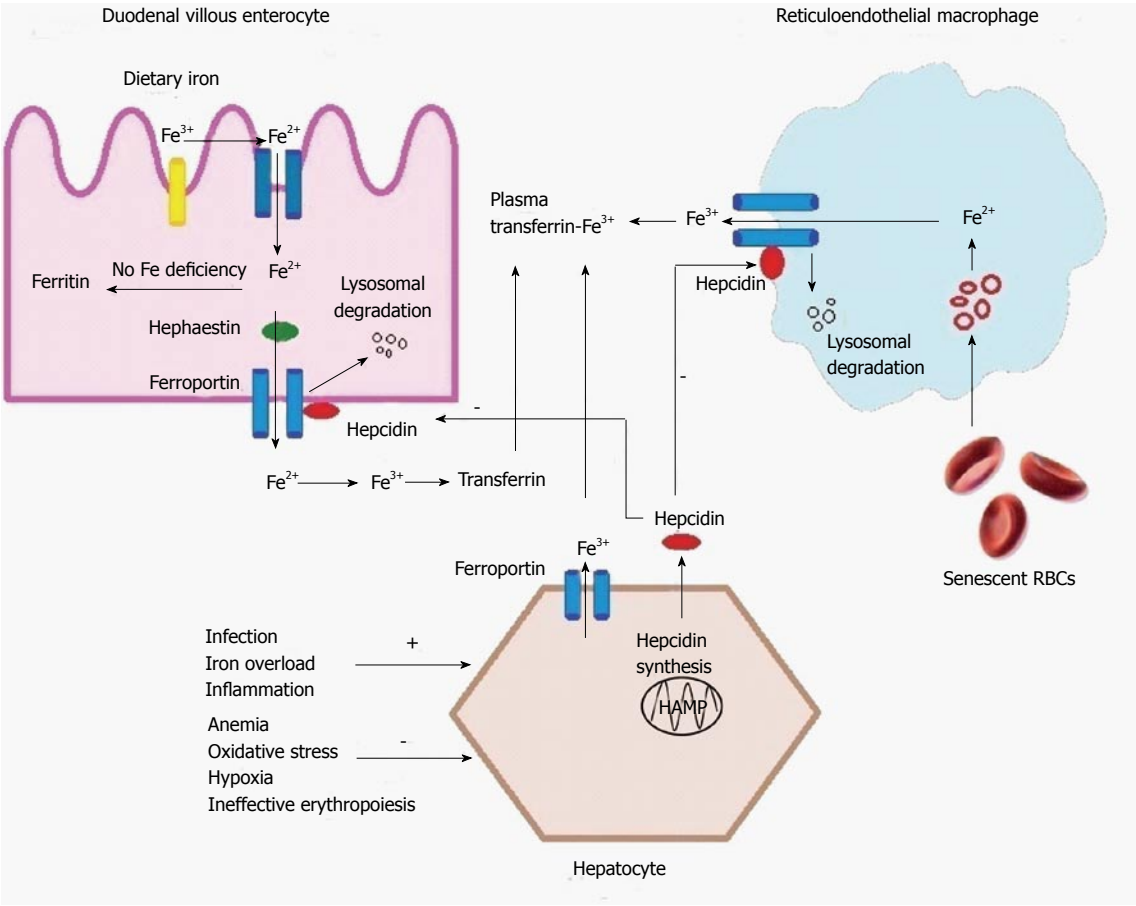

Figure 2 Role of hepcidin in the regulation of iron homeostasis. Fe^{3+} is reduced to Fe^{2+} by enterocyte brush border reductase Dcytb, transported across brush border membrane by DMT1. If iron demand is low, Fe^{2+} is stored as ferritin and sloughed with enterocytes. If iron demand is high, iron is oxidized by oxidase hephaestin and then exported into the plasma at the basolateral membrane by ferroportin[23]. Hepcidin binds to iron exporter, ferroportin 1, leading to its phosphorylation, internalization by binding to JAK 2 and lysosomal degradation, thus preventing iron release into the plasma[16]. DcytB: Duodenal ferric reductase; DMTI: Divalent metal transporter 1.

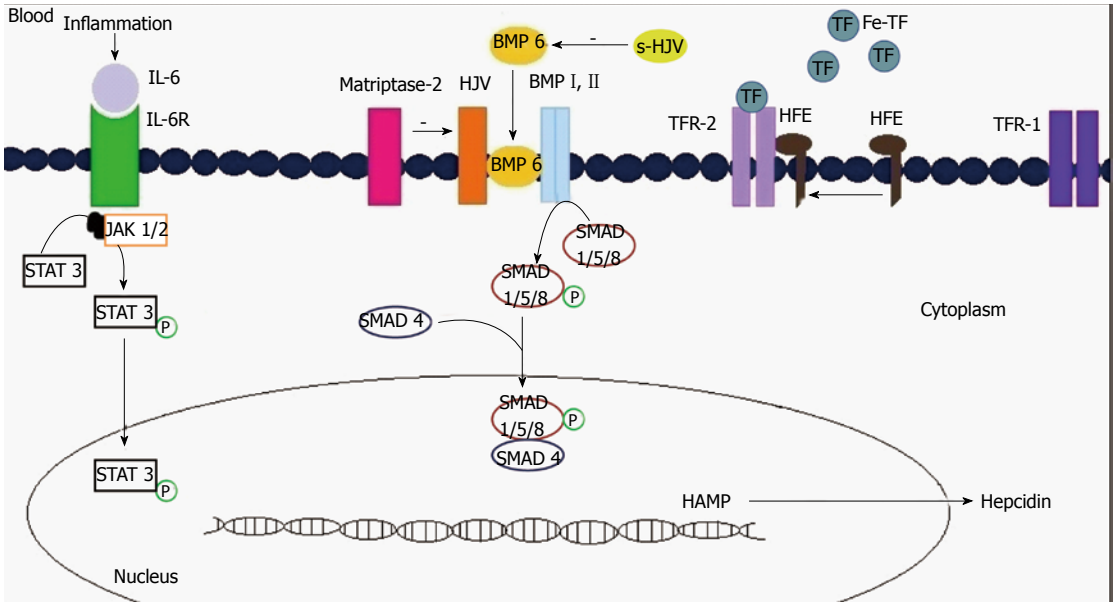

Figure 3 Signaling pathways regulating hepcidin expression in the liver. Enterocyte iron induces BMP6 expression. BMP6 is released *via* the portal vein to act on cell-surface receptors in the liver, BMPR-I, BMPR-II, and HJV, a co-receptor of BMP, leading to phosphorylation of cytosolic transcription factors, SMAD 1/5/8, which complex with SMAD 4[16,21]. This heteromeric complex translocates to the nucleus and enhances transcription of hepcidin gene, HAMP. In iron deficiency, HJV is cleaved by matriptase-2 activation reducing BMP signaling, and BMP is sequestered by s-HJV, preventing its interaction with plasma membrane HJV, decreasing hepcidin expression[21]. IL-6: Interleukin 6; BMP: Bone morphogenetic protein; HJV: Hemojuvelin; s-HJV: Soluble HJV; TfR2: Transferrin receptor 2; HFE: Hereditary hemochromatosis protein.

normal.

Serum transferrin carries Fe^{3+} in plasma and delivers iron from the sites of iron absorption (duodenal enterocytes and macrophages) to all tissues. Therefore, its level is higher in IDA. However, as it is an acute-phase protein, its level can be decreased during inflammation, despite normal or low iron stores.

Transferrin saturation (TSAT), an indicator of the iron load of circulating transferrin, gives an indirect measure of extent of iron utilization[16]. TSAT is the ratio of serum iron and total iron-binding capacity, multiplied by 100. It is decreased in both IDA and ACD. Pregnancy and oral contraceptives increase plasma transferrin levels; therefore, TSAT may be low in such patients, despite normal iron stores[16]. Hepcidin is increased during inflammation and decreased in IDA. It prevents iron absorption, causes retention of iron in the macrophages, and inhibits erythropoiesis.

Soluble transferrin receptor (sTfR) is a measure of erythropoietic activity. It is directly proportional to erythropoietic activity and inversely proportional to tissue iron availability[28]. sTfR is used to differentiate iron deficiency (increased sTfR and low serum ferritin) from inflammation (normal sTfR and serum ferritin) and to diagnose a combination of iron deficiency and inflammation (increased sTfR and normal serum ferritin)[24,27]. However, its use is limited due to its cost and unavailability in many laboratories.

The sTfR/log ferritin ratio (sTfR-ferritin index) may be an early indicator of depletion of iron stores[29]. A ratio < 1 suggests ACD and excludes iron deficiency, while a ratio > 2 suggests either IDA or mixed IDA and ACD[27].

Functional iron deficiency is the imbalance between the iron requirements of the erythroid marrow and the iron supply, when the body cannot supply iron rapidly enough to maintain an increased erythropoietic rate. This leads to reduced reticulocyte and erythrocyte cellular hemoglobin (Hb) content[30]. Reticulocyte hemoglobin content (CHr) and the percentage of hypochromic RBC are indicators of red cell hemoglobinization and, thus, functional iron deficiency, regardless of inflammatory states[30]. In IDA, CHr > 40 and hypochromic RBCs > 5%[31].

Iron binds to protoporphyrin IX to form heme. In the absence of iron, zinc binds to protoporphyrin to form zinc protoporphyrin (ZPP). ZPP indicates iron levels in the bone marrow during erythropoiesis and is unaffected by ACD or inflammation[16].

MANAGEMENT OF IRON DEFICIENCY ANEMIA IN IBD

Goals of therapy

It is important to realize that IDA commonly accompanies IBD. The treatment of IDA should not be overlooked. Iron supplementation should be started as soon as a patient is found to have iron deficiency anemia. If a patient has iron deficiency without anemia, the decision to initiate iron therapy depends on the clinical scenario and the patient's preference[24]. The treatment options *vs* frequent laboratory monitoring should be discussed with the patient. The decision to initiate therapy and the type of therapy is determined by symptoms, severity of anemia, IBD disease activity, comorbidities, and risks associated with therapy[24]. Anemia impairs quality of life, even in the absence of specific symptoms, in patients with IBD. Iron therapy leads to significant improvement in the quality of life (QOL)[32]. Therefore, the therapeutic goals of IDA are to normalize hemoglobin, serum ferritin, and TSAT levels, replenish iron stores (serum ferritin > 100 g/L), avoid the need for blood transfusions, and improve the QOL. Three treatment options are available for iron deficiency anemia in IBD: oral iron, parenteral iron, and erythropoietin[33].

Response to therapy

After starting iron therapy, an increase in reticulocyte count occurs within 2 wk, hemoglobin rises by 2 g/dL within 4 wk, and hemoglobin level returns to normal within 8 wk. Oral iron therapy should be continued for at least 6 mo after the hemoglobin has normalized, in order to replenish iron stores[34].

To assess the response to therapy, hemoglobin should be measured within 4 wk of the initiation of iron therapy. After 4 wk of iron therapy, response to treatment is considered appropriate or optimal, if hemoglobin rises by at least 2 g/dL; partial, if hemoglobin rises by 1-1.9 g/dL; absent, if hemoglobin rises less than 1 g/dL[24,35].

If the response to iron therapy is suboptimal, oral iron administration should be changed to intravenous iron therapy, erythropoietic agents should be added to intravenous iron therapy, or causes of anemia should be reassessed. A serum ferritin > 100 g/L indicates appropriate iron stores in a patient taking oral iron[24]. Serum ferritin is falsely high and is not useful for monitoring intravenous iron supplementation, in such cases a TfS > 50% indicates iron overload[36].

Oral iron therapy

Indications[16]**:** Anemia with hemoglobin > 10 g/dL, Quiescent or mildly active disease, in which oral iron absorption is not affected[37] in the absence of absolute indications for intravenous therapy (as mentioned below).

Advantages: There are some advantages, *e.g.,* convenience and inexpensive, in oral iron therapy.

Limitations[33]**:** (1) intolerance and non-compliance due to side effects: abdominal pain, nausea, bloating, diarrhea; (2) impaired absorption due to duodenal inflammation, intestinal resection, severe disease activity; (3) partial or incomplete absorption; (4) non-absorbed iron can be toxic and worsen disease activity in IBD as a result of oxidative stress, neutrophilic infiltration, increased cytokines, and activation of NF-

kappa B[38]; (5) experimental studies in animal models showed increased colon carcinogenesis with oral iron supplementation by inducing local oxidative stress at sites of active inflammation[39,40]; (6) slow response to therapy, cannot compensate for ongoing excessive blood loss; and (7) effective for short periods.

Available oral iron formulations: (1) ferrous fumarate has 106 mg elemental iron/tablet; (2) ferrous sulfate has 65 mg elemental iron/tablet; (3) ferrous gluconate has 28-36 mg iron/tablet; and (4) ferrous sulfate Elixir: 44 mg/5 mL (used if intolerant to oral iron tablets).

A maximum of 10-20 mg of oral iron can be absorbed per day. The recommended maximum daily dose is up to 100 mg elemental iron per day, as higher doses do not increase its absorption and efficacy, and the side effects of oral iron are dose-related[24,26]. Oral iron should be started at a low dose after meals. If well tolerated, the dose can then be increased and should be taken on an empty stomach to increase absorption. Iron should be given two hours before, or four hours after, ingestion of antacids. Iron is best absorbed as the ferrous (Fe^{++}) salt form in a mildly acidic medium, so a 250 mg ascorbic acid tablet or a half-glass of orange juice can be added at the time of iron administration to enhance the degree of iron absorption. Soy protein, dietary calcium, phytates (bran, oats, rye), cereals, tea, antacids such as H2 receptor blockers, and proton pump inhibitors prevent absorption of nonheme iron.

Intravenous iron therapy

According to the international consensus statement, the preferred route of iron supplementation in IBD is intravenous[24].

Indications[24,41]: (1) severe anemia, hemoglobin < 10 g/dL; (2) intolerance to oral iron therapy; (3) failure of oral iron therapy; (4) need for quicker and prolonged response; and (5) active disease (CRP > 5 mg/L).

Advantages[34]: (1) repletion of iron stores in unaffected by inflammation, intestinal resection; (2) rapid reversal of IDA; (3) relatively better tolerance and fewer side effects; (4) compliance can be monitored; (5) a single dose is sufficient for a few intravenous (IV) iron formulations (ferric carboxymaltose; low-molecular weight iron dextran); and (6) concurrent use of erythropoietin.

Limitations: (1) need for IV access and hospital staff for administration; (2) expensive; (3) inconvenience (travel, obtain IV access); and (4) iron dextran causes life-threatening anaphylactic reactions.

Available IV iron formulations: There are various IV iron preparations currently available for treatment of IDA. Their country-to-country availability, manufacturers, dosing, maximum and minimum infusion times, adverse reactions, costs, and FDA pregnancy category are provided in Table 3. The use of high molecular weight iron dextran is obsolete, due to the associated high risk of life-threatening anaphylactic reactions.

Low molecular weight (LMW)-iron dextran (Cosmofer, INFeD) is more efficacious than oral iron in significantly raising hemoglobin levels within 8 wk[42]. LMW-iron dextran was associated with IgE-mediated anaphylactic reactions in up to 5.7% of patients during test dose infusion in a case-matched study[42,43]. Therefore, a black box warning that a test dose is required prior to its infusion is now included in the package insert. Its administration is time-consuming and infusions can take up to 4-6 h.

Iron gluconate (Ferrlecit) is indicated for patients with chronic kidney disease (CKD) receiving hemodialysis and supplemental epoetin therapy. A test dose is not required prior to its use. It has benzyl alcohol as a preservative.

Iron sucrose (Venofer) is indicated for IDA in CKD patients and requires a test dose only in Europe. It has been widely used for IDA due to its efficacy, safety, and better tolerability[44]. But, its use has been limited, due to an increased number of applications, and its infusion can take up to 3.5 h.

Ferric carboxymaltose (Ferinject, Injectafer) has been studied in patients with iron deficiency of different etiologies, such as non-dialysis and dialysis-dependent chronic kidney disease (CKD), IBD, heavy menstrual bleeding, post-partum IDA, or patients with chronic heart failure and IDA. It has been shown to be efficacious and well-tolerated when compared with oral iron[45] and iron sucrose[46].

Ganzoni's formula is useful to estimate individual iron requirements[47]: Iron deficit (mg) = body weight (kg) x [target Hb-actual Hb (g/dL) x 2.4] + stored iron (500 mg).

However, it is inconvenient, inconsistently applied in clinical practice, and underestimates iron requirements[45]. A simplified method was used by the authors to calculate the cumulative iron dose (Table 4) for the ferric carboxymaltose group, instead of the traditional Ganzoni's formula used in the iron sucrose group[46]. Ferric carboxymaltose is administered in 1-2 infusions, with each infusion given one week apart. It can be infused in 15 min, thus increasing the compliance rate. The use of ferric carboxymaltose for IDA was shown to be more cost effective and convenient than iron sucrose[48]. Transient hypophosphatemia, without clinical symptoms, was observed in a clinical study[46].

Ferumoxytol (Rienso, Feraheme) is indicated for IDA in patients with chronic kidney disease (CKD). It has a rapid administration time (minimum of 17 s for 510 mg dose), with a second dose given in 3-8 d. However, the safety and efficacy of infusion of 1020 mg of ferumoxytol over 15 min has been demonstrated in a single arm, open-label trial conducted at one center[49].

Table 3 Available preparations for intravenous iron supplementation[16,24,26]

	Iron dextran (LMW)	Iron gluconate	Iron sucrose	Ferric carboxymaltose	Ferumoxytol	Iron isomaltoside
Product/Europe	CosmoFer®	Ferrlecit®	Venofer®	Ferinject®	Rienso®	Monofer®
Product/United States	INFeD®	Ferrlecit®	Venofer®	Injectafer®	Feraheme®	Not available in United States
Manufacturer	Pharmacosmos	Sanofi-Aventis	Vifor	Vifor	AMAG	Pharmacosmos
Test dose required	Yes	No	Yes (in Europe)/No	No	No	No
Maximum approved dose	20 mg/kg	125 mg	200 mg (500 mg in few countries) 7 mg/kg	Ferinject- 1000 mg, or up to maximum of 20 mg/kg Injectafer-1500 mg if patient's weight > 50 kg 15 mg/kg if < 50 kg	1020 mg	20 mg/kg
	Iron dextran (LMW)	Iron gluconate	Iron sucrose	Ferric carboxymaltose	Ferumoxytol	Iron isomaltoside
Maximum injectable single dose	100 mg (2 mL)	125 mg (10 mL)	200 mg (10 mL)	Ferinject- 1000 mg or up to maximum of 20 mg/kg Injectafer- 750 mg (15 mL)	510 mg (17 mL)	200 mg (2 mL)
Maximum infusion time	360 min (6 h)	30-60 min	210 min (3.5 h)	15 min	15 min	15 min
Maximum injection time	2 min	10 min	5-10 min	Bolus push over 7.5 min	17 s (1 mL/s)	Bolus push
Dose-related reactions	Dextran induced IgE -mediated anaphylaxis, hypotension, edema	Hypotension, edema	Hypotension, edema	None reported	None reported	None reported
Relative risk of adverse side effects	Moderate	Low	Very low	None reported	Very low	None reported
Costs per 500 mg in €[1]	84-86 €	52-56 €	105-100 €	170-175 €	82.12	170-175 €
Costs per maximum single injectable dose in United States dollars	$37.70	$76.32	$120	$993.75	$658.54	Not available
FDA Pregnancy category	INFeD-category C Cosmofer - no data available	B	B	C	C	Can be used in 2nd and 3rd trimester
Additional comments		FDA approved for IDA in CKD receiving hemodialysis and supplemental epoetin therapy	FDA approved for IDA in CKD patients	Has been studied in patients with IDA associated with CKD either dialysis and nondialysis dependant, IBD, CHF, post-partum and pregnancy patients. Transient hypophosphatemia has been reported	FDA approved for IDA in CKD patients May transiently interfere with "tissue" diagnostic ability of MRI for up to 3 mo and "vascular" MRI for up to 2 d	Very low immunogenic potential

[1]In Germany August 2012. Prescribing information of marketed products, package inserts. LMW: Low molecular weight; CKD: Chronic kidney disease; CHF: Congestive heart failure; IDA: Iron deficiency anemia; IBD: Inflammatory bowel disease.

Table 4 Determination of the Cumulative Iron Dose[46]

Hemoglobin (g/dL)	Body weight < 70 kg	Body weight > 70 kg
> 10	1000 mg	1500 mg
7-10	1500 mg	2000 mg

Ferumoxytol is composed of superparamagnetic iron oxide nanoparticles coated with a low molecular weight semisynthetic carbohydrate. This agent may transiently interfere with the tissue diagnostic ability of MRI for up to 3 mo, and with vascular MRI for up to 2 d, which is a limitation for patients with IBD who will need an MRI within 3 mo[50]. If MR imaging is required within 3 mo after ferumoxytol administration, T1- or proton density-weighted MR pulse sequences are used to minimize the effects of the agent, and the radiologist should also be notified.

Iron isomaltoside 1000 (Monofer) is the newest IV iron product, but it is not available in the United States. This agent has very low immunogenic potential and a very low content of labile and free iron, enabling

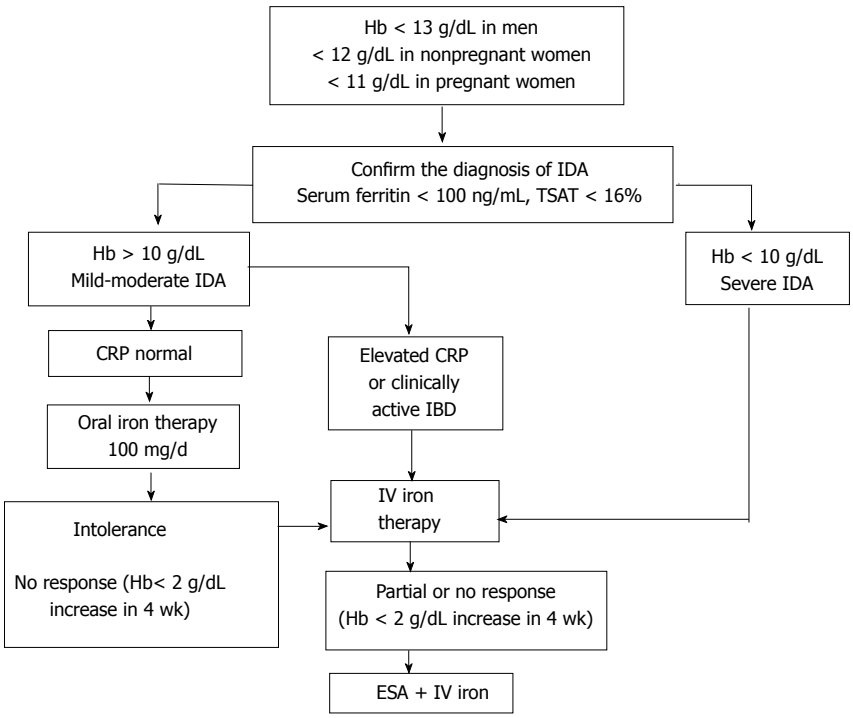

Figure 4 Management of iron deficiency anemia in patients with inflammatory bowel disease[16]. Hb: Hemoglobin; IDA: Iron deficiency anemia; TSAT: Transferrin saturation; CRP: C-reactive protein; IBD: Inflammatory bowel disease; IV: Intravenous; ESA: Erythropoiesis stimulating agents.

healthcare workers to administer a rapid high-dose infusion in doses exceeding 1000 mg in a single infusion, without the need for a test dose[51].

Erythropoiesis-stimulating agents

There is a component of anemia that is secondary to chronic inflammation in patients with IBD. Erythropoiesis-stimulating agents (ESA) are expensive and have risks associated with their administration. Therefore, these agents are recommended for treatment of anemia associated with IBD in patients who do not respond to IV iron therapy, when immunosuppressive therapy has not suppressed inflammation, and for patients requiring blood transfusions[52].

When patients are treated with ESAs, functional iron deficiency develops. Functional iron deficiency refers to the failure of iron supply or transport and insufficient availability of iron for erythropoiesis, despite normal body iron stores. Therefore, ESAs are used in combination with IV iron therapy[52]. The increased hepcidin levels in ACD prevent oral absorption of iron. Therefore, IV iron supplementation should be given instead of oral iron.

Blood transfusions

The need for red blood cell transfusion depends on the rate of bleeding, hemodynamic stability, hemoglobin level, and comorbidities. It is not a substitute for treatment of IDA with oral or IV iron supplementation. Its use in chronic anemia should be avoided, due to potential side effects and cost[24].

CONCLUSION

IDA is the most common anemia in IBD. Therefore, anemic patients with IBD should be evaluated for IDA. IDA mandates adequate and appropriate treatment, as it influences the patient's quality of life and morbidity. It should be considered as an extraintestinal manifestation of IBD. The basic workup may be insufficient to diagnose IDA in the presence of inflammation. Table 2 summarizes the laboratory parameters necessary to differentiate IDA from ACD. Recent clinical trials support IV iron therapy as a preferable option, especially in clinically active IBD and moderate-severe IDA. IV iron therapy was shown to have better tolerability and efficacy, with fewer infusions, a better safety profile, and, accordingly, improved patient compliance. Figure 4 summarizes the management of IDA in IBD[16]. The ultimate goal is to normalize hemoglobin levels.

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MINIREVIEWS

Designer probiotics: Development and applications in gastrointestinal health

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Abstract

Given the increasing commercial and clinical relevance of probiotics, improving their stress tolerance profile and ability to overcome the physiochemical defences of the host is an important biological goal. Herein, I review the current state of the art in the design of engineered probiotic cultures, with a specific focus on their utility as therapeutics for the developing world; from the treatment of chronic and acute enteric infections, and their associated diarrhoeal complexes, to targeting HIV and application as novel mucosal vaccine delivery vehicles.

Key words: Gastrointestinal tract; Probiotics; BetL; *Listeria monocytogenes*; Bifidobacterium; Lactobacillus

Core tip: Genetically engineered probiotic bacteria, with improved *in vivo* stress survival and persistence, have the potential to enhance, and in some instances replace, conventional prophylactic and therapeutic measures. This is particularly relevant in the developing world, where chronic and acute infections, and their associated sequelae impose a significant clinical and economic burden.

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INTRODUCTION

Probiotics are commensal organisms that can be harnessed for therapeutic benefit[1]. In acute infections probiotics may enhance protection mediated by commensal flora through direct antagonism, *i.e.*, completion for niches and nutrients, or *via* the production of antimicrobials, such as bacteriocins[2]. In chronic clinical conditions, such as immuno-suppression, microbe-host signalling is likely more relevant to effective probiotic function. This bacterial-host dialogue within the gut lumen, functions to maintain an effective mucosal barrier while also priming the host for further

responses to injury[3]. These health promoting benefits of probiotics, coupled with fact that they are cheap to produce, transport and store, makes them an attractive alternative to traditional therapies, especially in underdeveloped and war-torn territories.

Herein, I review key milestones in the development of probiotic based therapies, focusing particularly on problems encountered in developing countries such as acute and chronic enteric infections, as well HIV (AIDS) which continues to devastate sub-Saharan Africa[4]. Little or no access to appropriate medical care, often coupled with compromised immunity means that the malnourished are significantly more predisposed to infections by enteric pathogens, leading to incapacitating and dehydrating diarrhoea which in turn leads to a dramatic worsening of an already severely compromised nutritional status. Superimposing the HIV (AIDS) pandemic on an already distressed situation has created a state of affairs which needs to be urgently addressed[5].

Probiotic therapy, specifically the use of engineered probiotic strains, is a viable alternative to traditional approaches to alleviate suffering, to fight existing diseases and to protect against future infections[6]. Herein, I review the current state of the art in the design and application of probiotic cultures as therapeutics for the developing world.

Probiotics as therapeutics for the developing world

Almost a third of the world's population are malnurished[7], a quarter of which are children in the developing world. Malnutrition significantly reduces cell-mediated immunity, immunoglobulin A (IgA) concentrations and cytokine production[7]. This in turn leads to an increased risk of infection, accompanied by bouts of acute and recurrent diarrhoea – which further exasperates an already depressed nutritional status. Indeed, almost half of all diarrhoea-related mortalities are linked to malnutrition[8], while morbidity is also disturbingly high (approximately 4 times more episodes per child per annum in developing countries than in the developed world). Diarrhoeal disorders exists as either acute diarrhoea; associated with sudden onset infections, characterized by recovery within two weeks, and chronic diarrhoea which lasts more than two weeks and usually arises as a symptom of malnutrition or immunodeficiency[9].

Probiotic bacteria have been shown to considerably limit the incidence and duration of diarrhoea associated with both acute infectious illness and chronic episodes linked to malnutrition[10]. Shornikova *et al*[11] showed that *Lactobacillus reuteri* can reduce the duration of acute diarrhoea in infants by one full day. Guandalini[12] observed similar effects with *Lactobacillus rhamnosus*, which also decreased the duration of hospital stays. Furthermore, in addition to alleviating the symptoms of malnutrition and diarrhoea, probiotics have also been used to specifically target bacterial and viral pathogens[13-16]. *Lb. casei* Shirota, for example was shown by Ogawa *et al*[17] to reduce *Escherichia coli* O157:H7 colonization, while Pascual *et al*[18] observed complete exclusion of *Salmonella enteritidis* by *Lb. salivarius.* Furthermore, even more impressive effects have been observed with mixed probiotic cocktails. Casey *et al*[19] reported significant amelioration of clinical symptoms of *Salmonella* Typhimurium infection in pigs using *LIVE5*; a cocktail of two *Lactobacillus murinus* strains with one strain each of *Lb. salivarius* subsp. *salivarius*, *Lb. pentosus* and *Pediococcus pentosaceous*. Pigs administered this mixture exhibited significantly lower levels of *Salmonella* infection, reduced frequency, severity and duration of diarrhoea, and enhanced weight gain relative to animals fed on a skim milk placebo. Nisbet *et al*[20] observed similar decreases in *Salmonella gallinarum* mediated mortality using a commercial probiotic mixture, while Johnson-Henry *et al*[21] showed that a *Lactobacillus* mixture reduced inflammation in *Helicobacter pylori*-infected animals. Furthermore, clinical trials in colonized humans revealed significantly lower levels of *H. pylori*, and decreased adverse side effects[22]. Probiotics are also effective against rotavirus, an enteric virus which accounts for approximately 60% of all diarrhoeal episodes in developing countries[23,24]. Specifically, *Lactobacillus casei* subsp. *casei* strain GG (LGG) has been shown to stimulate a rotavirus-specific IgA antibody response, which may confer immunity against future rotavirus infections[25].

However, one of the most significant limitations in the clinical application of probiotics is that the most clinically relevant probiotics are often the most physiologically fragile. Improving probiotic stress tolerance is thus a biological imperative. Below we describe the application of the "Patho-biotechnology" concept for the development of improved probiotic cultures[26-29] (Figure 1).

DEVELOPMENT AND APPLICATION OF PROBIOTIC THERAPEUTICS

Fluctuations in temperature and water availability (a_w) are the most common stresses associated with food production[30,31]. The ability to overcome these stresses is thus an important criterion in the selection of commercially viable and clinically effective probiotics[32]. A common strategy to overcome temperature and osmotic stress is the accumulation of compatible solutes, such as the plant derived trimethyl ammonium compound glycine betaine[33], which serves to stabilise cellular function under stressful conditions[34].

Improving compatible solutes accumulation is thus an important first step in the development of more physiologically robust probiotic strains[35]. Several sophisticated mechanisms for compatible solute accumulation have evolved over time[34]. Indeed, the intracellular pathogen *L. monocytogenes*, which serves as a useful model for Gram positive osmotolerance[36], has three compatible solute uptake systems (BetL, Gbu and OpuC[37]), the first to be identified being the secondary betaine transporter BetL[38-40]. By cloning

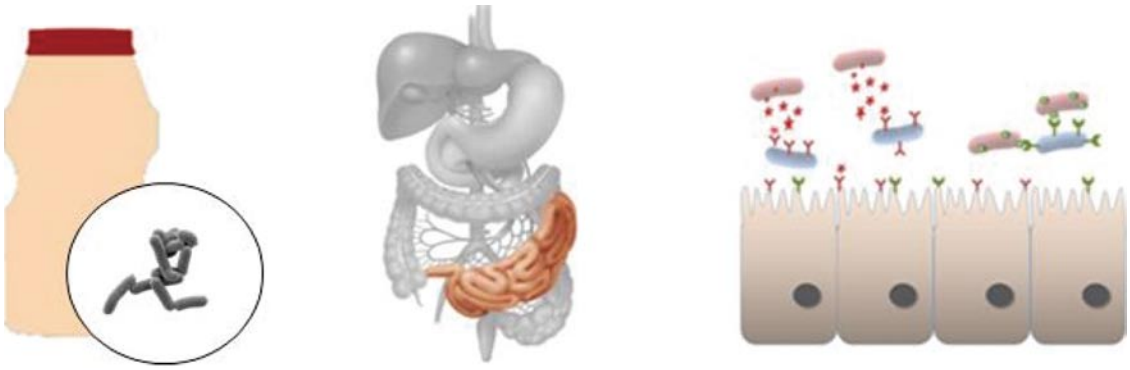

Figure 1 Overview the patho-biotechnology concept; enhancing probiotic delivery in the food matrix, gastrointestinal persistence and clinical efficacy.

betL downstream of the nisin inducible promoter P*nisA*, we were able to assess the ability of BetL to contribute to probiotic survival under a variety of stresses[41,42]. As expected, the *Lb. salivarius* strain heterologously expressing BetL exhibited a significant increase in betaine uptake compared to the wild type, untransformed control. Indeed, the increased betaine uptake was sufficient to confer improved resistance to chill and cryotolerance, freeze-drying, spray-drying and barotolerance[41,43-46].

In addition to *ex vivo* stresses, probiotic bacteria must also overcome the *in vivo* defences of the host[30,31,47,48]. We demonstrated heterologous expression of BetL in *Bifidobacterium breve* UCC2003, significantly improved survival of the probiotic in gastric juice[49]. In support of this, Termont *et al*[50] also reported similar effects in a *L. lactis* strain expressing the *E. coli* trehalose synthesis genes, suggesting a novel protective role for compatible solutes in the gastric environment. Furthermore, we have reported roles for carnitine and proline in contributing to bacterial gastrointestinal survival[51-54]. Upon exiting the stomach, bacteria enter the upper small intestine where they are exposed to elevated osmolarity (equivalent to 0.3 mol/L NaCl). As was observed with *L. salivarius*[41], a significant osmoprotective effect was evident following BetL expression in *B. breve*, facilitating growth of the probiotic in conditions similar to those encountered *in vivo*. Furthermore, whilst stable colonisation of the murine intestine was achieved by oral administration of *B. breve* UCC2003, strains expressing BetL were recovered at significantly higher levels than the parent in the faeces, intestines and caecum of inoculated animals. Additionally, BetL significantly improved the clinical efficacy of the probiotic; resulting in significantly reduced levels of systemic infection following oral inoculation with *L. monocytogenes*, compared to the control.

In addition to improving physiological stress tolerance, "designer probiotics" have been designed to specifically inhibit enteric infections by blocking ligand-receptor interactions between the pathogen and/or secreted toxins and the host[55]. Blocking receptor binding prevents infection, while toxin neutralization dampens clinical symptoms. Engineered to express receptor-mimic structures on their surface[56], orally administered probiotics neutralize toxins and inhibit pathogen adherence to the intestinal epithelium. Examples of such constructs include an *E. coli* strain expressing a chimeric lipopolysaccharide terminating in a shiga toxin (Stx) receptor, which binds to and neutralises Stx1 and Stx2[56], as well those with receptor blocking potential against cholera toxin (Ctx) and Enterotoxigenic *E. coli* (ETEC) toxin LT[57,58].

As well as treating enteric infections, "designer probiotics" have also been enlisted to target HIV. Every day approximately 14000 people become infected with HIV, a majority of which are in developing countries. Rao *et al*[59] recently described the construction of an *E. coli* strain engineered to secrete HIV-gp41-haemolysin A hybrid peptides, which block HIV fusion and entry into target cells. This "live microbicide", administered either orally or *via* rectal suppository, colonizes the gut mucosa creating a protective barricade against HIV infection for four weeks[60]. Other anti-HIV probiotics include a human vaginal isolate of *Lactobacillus jensenii* engineered to secrete two-domain CD4 which inhibits HIV entry into target cells and *Streptococcus gordonii* modified to produce cyanovirin-N, a HIV-inactivating protein originally isolated from cyanobacterium[61].

PROBIOTICS BASED PROPHYLAXIS

The most effective prophylaxis for infectious diseases is vaccination; resulting in the mobilisation of an immune response capable of specifically targeting invading pathogens[29]. In addition to the classical approach to vaccination, involving induction of acquired immunity to specific antigens, there is a growing awareness of the importance of innate immunity, associated primarily with our commensal microflora[2,62-64]. Indeed, optimal development and functioning of the mucosal immune

response is dependent on microbial exposure early in post-natal life[65]. In the absence of such stimulation, development of the intestinal-associated lymphoid tissue is stunted and immune responses are suboptimal[66].

The development of efficient vaccine delivery vehicles remains challenging since traditional vaccines are typically based on either recombinant proteins or killed whole pathogens which, although safe, typically induce only weak immunity[67-69]. The alternative is to use viable or attenuated pathogens[15]. However, while this approach improves targeted delivery, it carries with it the possibility of reversion to virulence[70]. Using a patho-biotechnology based approach, probiotics are being engineered to function as novel vaccine delivery vehicles which, lack the possibility of reversion to virulence, and effectively stimulate both the innate and acquired arms of the immune response[68,69]. In line with this approach, Guimarães *et al*[71] engineered a *Lactococcus lactis* strain capable of delivering either DNA or protein into the epithelial cells of the small intestine. Heterologous expression of *inlA*, encoding a listerial eukaryotic cell adhesion factor, converted the otherwise non-invasive *L. lactis* strain into a safe and effective vaccine delivery platform. Furthermore, the addition of *hlyA* (encoding listeriolysin) to *L. lactis inlA*$^+$ enables phagosomal escape within the macrophage allowing MHC Ⅰ and Ⅱ stimulation[72].

Mucosal vaccine delivery, achieved using probiotic delivery platforms, has significant medical and methodical advantages, particularly for use in the developing world, including: reduced pain and the possibility of cross contamination associated with intramuscular injection, no requirement for expensive medically trained personnel and no cold chain requirement - a significant advantage in the tropical climes most often associated with the developing world.

CONCLUSION

While conventional medical research continues to provide effective prophylactics and therapeutics, these often remain beyond the reach of the developing world. In this context, probiotics provide a viable and cost effective alternative to fighting infection, modulating the immune response and alleviating the symptoms of malnutrition and its associated sequelae, all of which will ultimately contribute to health and social gain, particularly in the developing world.

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Human microbiome: From the bathroom to the bedside

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Abstract

The human gut contains trillions of bacteria, the major phylae of which include *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Fecal microbial transplantation (FMT) has been known of for many years but only recently has been subjected to rigorous examination. We review the evidence regarding FMT for recurrent *Clostridium difficile* infection which has resulted in it being an approved treatment. In addition there is some evidence for its use in both irritable bowel syndrome and inflammatory bowel disease. Further research is needed in order to define the indications for FMT and the most appropriate method of administration.

Key words: Fecal microbial transplant; Clostridium difficile; Side-effects; Indications; Metabolic disorders

Core tip: Fecal microbial transplantation is approved for the treatment of recurrent *Clostridium difficile* infection by either nasojejunal administration or colonoscopy. In addition there is some evidence for its use in both irritable bowel syndrome and inflammatory bowel disease. There are, however, reports of side effects including weight gain, diverticulitis and development of autoimmune disease. Treatment for non-approved conditions should be performed in the framework of clinical research trials in order to better define the indications.

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INTRODUCTION

The human microbiome is defined as the collection of organisms and their genomes inhabiting locations both in and on humans. Our understanding of the gastrointestinal microbiome (GIMb) has been assisted by the Human Microbiome Project[1] and the Metagenomics

of the Human Intestinal Tract investigational groups.

Trillions of bacteria are present in the human gastrointestinal tract and encompass from 2000 to 4000 different species of bacteria, both aerobic and anaerobic. The major phyla include *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*.

The human GIMb is in many ways an additional organ of the body. It has ontogeny, anatomy and physiology and its function may be disturbed in certain pathological conditions. It is possible in some instances to restore normal GIMb function by fecal microbiota transplantation (FMT).

Although the concept of fecal transplantation has become more widely practiced in recent years, it has a long history. More than 2000 years ago Ge Hong used FMT to treat food poisoning and severe diarrhea[2]. Fabricius of Acquapendente in the 16th century described the transplantation of enteric bacteria. The first report of the use of FMT in recent times in more traditional medicine was that of Eiseman *et al*[3] in the form of a fecal enema obtained from a healthy donor in 4 cases of pseudomembranous colitis.

It is the purpose of this paper to review the current state of fecal transplantation.

FMT FOR CLOSTRIDIUM DIFFICILE INFECTION

Clostridium difficile infection (CDI) is a gram positive anerobic bacillus that produces spores. It is present in the bowel of 4%-13% of asymptomatic people[4-6]. CDI is an increasingly recognized cause of infectious hospital-acquired diarrhea in the developed world[7]. In healthy people it has been thought that it lacks the potential to produce toxins which can result in diarrhea. However, there is an increasing recognition of *C. difficile* in children, healthy adults and pregnant women[8-10]. Treatment consists of antibiotic therapy with metronidazole, vancomycin or fidaxomicin[11]. About 25% of those suffering from CDI have a recurrence after the first course of treatment[12]. For those patients with a recurrent episode of infection, there is a 40% chance of experiencing another recurrence and for those who have had more than 2 episodes there is a 60% chance of a further episode[12].

In view of the need for a more effective treatment for recurrent CDI, the use of FMT has been examined. In modern clinical research, there is usually an hypothesis that is examined in laboratory animals and then tried in placebo-controlled double blind clinical trials. However, for FMT as treatment for recurrent CDI this order has been reversed. The success of FMT for recurrent CDI has been demonstrated in case series[13-15] and one randomized controlled trial[16].

Hamilton *et al*[13] have reported their experience with 43 consecutive patients treated with FMT for recurrent CDI at the University of Minnesota from 2009. The FMT was performed by colonoscopy. Fourteen of these patients had inflammatory bowel disease (IBD). There was a mean of 5.9 relapses and the mean success rate was 86%.

Mattila *et al*[14] reported a retrospective review of 70 patients from 5 medical centers in Finland from 2007-2010. The FMT was performed by colonoscopy. Thirty six (51%) of the patients had the 027 ribotype strain that is more virulent and associated with a higher rate of relapse. All of the 34 patients with the non-027 strain had a resolution within 12 wk compared to 32 of 36 patients (89%) with the 027 ribotype. The four nonresponders had serious comorbidity. Four of the patients who responded experienced a relapse after a year. Two were cured by repeat FMT and 2 by repeat antibiotic treatment.

Brandt *et al*[15] reported the multi-center United States experience of FMT for recurrent CDI. There were a total of 94 patients of whom there was follow-up data in 77. The primary cure rate was 91%. The secondary cure rate for the 7 patients that did not respond or relapsed was 98%. The mean follow-up period was 17 mo and was up to 68 mo.

There has recently been published a randomized controlled open-label trial of patients with at least one episode of recurrent CDI from Holland and Finland. The numbers of patients involved was small-13 in each of three groups. The groups were randomized to receive initial vancomycin for 4 d and then either bowel lavage, bowel lavage and donor feces through a nasoduodenal tube or just vancomycin alone. The study was stopped prematurely after an interim analysis revealed a resolution of CDI of 81% in the group receiving feces by nasoduodenal infusion as compared to 31% receiving vancomycin alone and 23% receiving vancomycin with bowel lavage[16] (Figure 1). These were much smaller numbers than initially planned for. Thus this study is consistent with previous case reports.

In view of the limited data from randomized controlled trials a systematic review of FMT involving 317 patients from 27 case series and reports has been published[17]. Two thirds of these were case series. There was resolution of the disease in 92% of cases, 89% after a single treatment. The lowest rate of resolution was by infusion *via* gastroscopy or nasojejunal tube. This is especially relevant regarding the above mentioned randomized trial[16], since the number of cases was very small due to the early termination of the trial mandated by the review committee. Although there is great heterogeneity between the various case reports and case series making up this systematic review, there was found to be a higher relapse rate in patients who received both bowel lavage and antibiotics before FMT (4/33, 12.1% *vs* 5/150, 3.3%).

In the light of this data FMT is now recognized as treatment for a third recurrence of CDI[18].

The rationale for use of FMT has been shown in a mouse model of mice treated with clindamycin and then infected with *Clostridium difficile* that had been

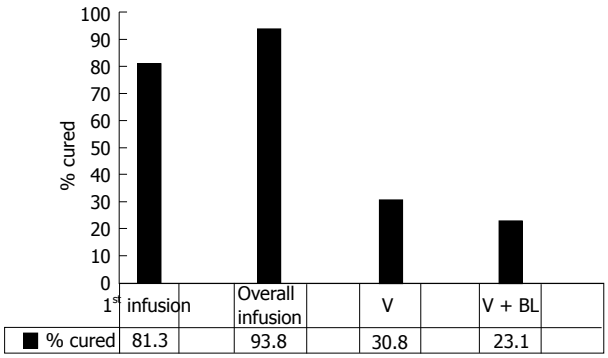

Figure 1 Rates of cure after treatment of recurrent *C. difficile* infection by nasojejunal infusion of donor feces. The data show first infusion, overall infusion after retreatment for a treatment failure, vancomycin (V) treatment, and vancomycin treatment and bowel lavage (V + BL) (16).

isolated from patients with CDI. The mice developed chronic disease and responded to the administration of homogenized feces from healthy mice[19]. Thus the data that is available support the use of FMT for the treatment of recurrent CDI.

FMT - HOW TO DO IT

The first issue to be addressed is the donor of the feces. Initially the donors of the feces were "healthy donors" with no other details provided[3]. In some case the stools of medical residents were used. Until 2011, a partner or family member was the most frequent source of donor. It was assumed that any infectious diseases would already have been transmitted between the donor and recipient. This has not been examined by evidence-based medicine. More recently the NIH in the United States has required that donor stool be examined for C difficile toxin, enteric bacterial pathogens (including *Listeria monocytogenes, Vibrio cholera* and *Vibrio parahemolyticus*), parasites including Giardia (*via* antigen test), Cryptosporidium (antigen test), Isospora (acid-fast stain) and Rotavirus. In addition the donor blood needs to be screened for hepatitis A (IgM), B (HBsAg, anti-HBc-IgG and IgM, and anti-HBsAg) and C (HCV antibody) viruses, HIV type 1 and 2 and syphilis. In addition *H. pylori* should be tested for. The Israel Ministry of Health protocol is shown in Table 1. The tests for the donor may not be entirely covered by the medical insurance organizations since they may not be indicated for the routine medical care of the stool donor.

There is a growing trend for DIY stool transplants with instructions being available on the Internet- for example www.thepoweerofpoop.com. There are cases when patients refer themselves to centers performing FMT after self-administration of unscreened donor stool from a family member has failed[20]. The only systematic review of FMT suggested that stool from a related donor (spouse or intimate partner) resulted in a higher rate of cure (93.3%) as compared to an unrelated donor (84%).

The FMT program at the University of Minnesota has reported their experience with frozen/thawed or fresh fecal preparations from universal donors[13] with cure rates of 90% for frozen material and 92% for fresh material. This is higher than those reported with patient selected donors (70%), although only small numbers of patients were included in each group- 10 individual donors and 33 standard donors. A recent report of increase in weight after fecal transplantation from a related obese donor provides a note of caution[21]. For these reasons we believe that the use of individual donors should not be the first choice for obtaining feces for transplantation.

The donor needs to provide the stool sample into a clean plastic container. The amount is variable although 50 g in 250 mL of diluent is a common method. Different fluids have been used as the suspending fluid, including milk, water and saline. The resolution rates with saline and milk are 86.2% and 88.6%, with recurrence rates of 3.0% and 3.2%[17]. The use of water resulted in a resolution rate of 98.5% and recurrence of 7.8%. Three hundred milliliter is the usual dose for colonic FMT and 60 cc for upper GI tract FMT.

The patients who will receive the FMT need to have a large volume colonic lavage prior to the procedure. This is thought to cleanse the spores of *C difficile* that are responsible for the recurrence of the infection. There are different variations and some reports include loperamide if receiving a fecal enema[22], and proton pump inhibitor (to reduce the bactericidal effect of gastric acid) if the infusion of stools is *via* a nasogastric or nasojejunal route[23,24].

Some groups maintain the patient on vancomycin orally until the time of the FMT[16,25]. This practice is thought to reduce the vegetative forms of *C. difficile* since vancomycin has no action on the spores. However, the systematic analysis of Gough *et al*[17] found a higher relapse rate with the combination of bowel lavage and antibiotics. Our group policy is to discontinue antibiotics two days prior to FMT.

Recently, there has been a report of the use of capsules containing frozen stool. Twenty patients with recurrent CDI were treated with 15 FMT capsules daily for 2 d. The resolution rate was 70%. The 6 failures were retreated and 4 of these had resolution, resulting in an overall response rate of 90%. A total of 30% of the patients experienced mild abdominal complaints that resolved within 72 h. This is a preliminary study that needs to be repeated and expanded but is promising and if successful will probably replace the current methods of administration of the donor stools.

SAFETY AND COMPLICATIONS

Two studies have provided information regarding long-term follow up after FMT. A study from Finland of 70 patients had no data on complications[14], although the authors state "none of our patients had any serious adverse effects that could be related to fecal transplantation". A multi-center North American study

Table 1 The Israel Ministry of Health protocol for donor screening for fecal microbial transplantation

Patient eligibility for FMT
1 Questionnaire to exclude potentially transmissible diseases
(1) Practices protected sexual intercourse (or in a monogamous relationship with a healthy partner)
(2) Has not had tattooing in the previous six months
(3) Does not have any known infectious diseases
(4) No drug abuse
(5) Has not travelled to the Far-East, India, Africa, Central or South America within the previous six months
(6) Has no known autoimmune disease
(7) There is no history of any gastrointestinal disease, including inflammatory bowel disease, celiac disease or irritable bowel disease
(8) Has not had any previous bowel resection
(9) There is no history of infectious diarrhea in the previous 12 mo
(10) Has not received antibiotic therapy in the previous 2 mo
2 Laboratory tests
(1) Negative stool culture
(2) Negative stool microscopy, including Giardia, Cryptosporidium and *Isospora beli*
(3) Negative *Clostridium difficile* toxin
(4) Negative serological testing for HIV, HBV, HCV, HAV, VDRL, CMV
(5) Negative test for *Helicobacter pylori* (either C^{13} urea breath testing, stool antigen or serum antibodies)

FMT: Fecal microbial transplantation. HCV: Hepatitis C virus; HBV: Hepatitis B virus; CMV: Cucumber mosaic virus; VDRL: Venereal disease research laboratory; HIV: Human immunodeficiency virus.

reported data from more than 3 mo follow up in 77 of the 94 eligible patients. Ninety-seven percent of the patients stated that they would choose FMT again as treatment for a recurrent CDI and 53% would prefer it as a first option. Twenty-seven percent of the patients developed abdominal pain following FMT but it was minor and resolved within 10 d. Four patients developed an autoimmune disease-peripheral neuropathy, rheumatoid arthritis, Sjogrens and thrombocytopenia[15]. We have recently treated a patient with severe CDI who required 2 consecutive FMTs and developed thrombocytopenia after each transplantation. Recently, there has been a report of diverticulitis following FMT for CDI[26].

The field of FMT is rapidly developing and as experience accumulates there are more reports of possible side effects or complications. Thus it is important to carry out these procedures in centers with approved protocols and to discourage patient or non-physician supervised self-administration.

CDI is common in patients who are immunosuppressed. Recently a retrospective study from 16 medical centers in the United States reported their experience in FMT in 80 immunosuppressed patients with severe or recurrent or refractory CDI[27]. The majority of the patients were immunosuppressed due to solid organ transplantation or treatment for IBD. The cure rate was 79% for the first time and 89% overall. There was a 15% incidence of serious adverse events within 12 wk. Five of the 36 IBD patients had post-FMT disease flare. Thus it appears that successful FMT is possible in immunosuppressed patients, although with a slightly reduced success rate and a higher rate of adverse events.

OTHER USES FOR FMT

IBD

FMT appears to be an established treatment for CDI and attention has focused also on IBD. Gastrointestinal microbiome dysbiosis has an important role in the pathogenesis of IBD[28]. In addition a recent study employing the molecular biology technique of terminal restriction fragment length polymorphism (T-RFLP) to profile the bacterial species in fecal samples has enabled the calculation of a discriminant score which was shown to be a biomarker for disease activity in UC[29]. The first FMT for ulcerative colitis (UC) was reported in 1989[30] and described the reversal of the UC that Bennet (one of the authors of the paper) had suffered from for 7 years by FMT administered as fecal enemas. Case series have suggested some role for FMT in UC[31-33]. However, a recent report[34] of a prospective study of FMT in 5 adult patients with moderate to severe UC who had failed various immunosuppressive therapies adds a note of caution. None of the patients were receiving concomitant immunosuppressive therapy. None of the patients entered clinical remission after 12 wk of follow-up and only one had some clinical improvement. Additionally all of the 5 patients had fever and an elevation of CRP after the FMT and a worsening of the diarrhea the day after the procedure. In the patient who experienced some response there was an alteration of the fecal flora after FMT. Another recent trial of FMT for UC did not demonstrate a significant effect[35] in 6 patients but there was a change in the gut microbiota. The alteration of gut microbiota was temporary and it may be necessary to undergo repeated transplantation in order to maintain the altered gut microbiota. A phase 1 trial of FMT for 9 pediatric UC patients with mild-to-moderate activity reported no serious adverse effects and found that 7 of these (79%) had responded within a week[36]. It may be that certain population subgroups suffering from UC will derive benefit from FMT. A recent systematic review and meta-analysis of FMT therapy for IBD showed a clinical remission of 22% for UC[37].

Crohn's disease (CD) has also been treated by FMT. The data are limited to case reports and small case series[32,38]. The recent systematic review and meta-analysis of FMT as therapy for IBD found higher pooled estimate of clinical remission for CD, 60.5% as compared to UC 22%[37]. There have been 2 previous systematic reviews of FMT for IBD[39,40]. The success rate of FMT for adult IBD patients was 77.8%[39] but outcomes were measured by "success rates" only. There were also other methodological problems[37]. The other review[40] noted endoscopic and histologic remission of 63% in 24 patients but this too has been criticized for methodological flaws[37]. A note of caution is necessary following the report of bacteremia after FMT in a patient

with Crohns and CDI[41].

Thus the jury is out regarding the utility of FMT for IBD and further work is required in order to define the effect of different methods of delivery, changes on the microbiome and the interaction between phases of the illness (induction or maintenance) and the impact of additional therapies.

Irritable bowel disease

There is evidence linking dysbiosis to irritable bowel disease (IBS)[42,43] and thus there is a question regarding the possibility of FMT for treatment. There have been reports of a favorable outcome after FMT in diarrhea - predominant IBS[32]. A recent report of single-center experience of 13 patients with IBS of whom 9 had diarrhea-predominant, 3 constipation-predominant and one mixed-type[44], found resolution or improvement in symptoms in 70% of the patients overall. Presumably, the small sample size prevented the reporting of the response rate in each group separately. This subject has recently been reviewed[45]. There is a need for further research to define the role of FMT in the treatment of this common condition.

FMT and metabolic disorders

It is now apparent that there is an interaction between the microbiome of the intestinal tract and the metabolism of the human host and that there is a link to obesity[46]. Although there are reports of changes in the ratio of *Firmicutes/Bacteroides* with human obesity[47] other groups have not found such changes[48,49]. There are reports in mice of the induction of a phenotype of the metabolic syndrome *via* fecal transplants[50]. This complex subject has been reviewed[46,51,52]. Furthermore in mice the intestinal microbiota plays a role in the development of non-alcoholic fatty liver disease[53]. Recently stools from twins discordant for obesity has been shown to promote or impair the development of obesity in adult male germ-free mice[54].

In humans there is a single study reporting that FMT using stool from lean donors improves insulin sensitivity in obese male individuals concomitant with an increase in butyrate-producing intestinal bacteria[55].

We are currently conducting a randomized controlled trial of FMT in obese individuals undergoing screening colonoscopy in order to determine if there is a clinical effect on obesity in humans (clinical trials.gov NCT02336789).

Other conditions

Immune thrombocytopenia (ITP) was reported to be reversed in a patient treated with FMT for UC - but only published in an abstract form[55]. Other fields include autoimmune disease, allergic disorders, and neuropsychiatric disorders. This exciting field has recently been reviewed[56].

In summary, fecal microbial transplantation is now prime time treatment for refractory *Cl. difficile* infection. In addition, it may be of use for treating other disorders of the gastrointestinal tract including irritable bowel syndrome and IBD. Further research is needed to define the optimal method of administration of the stools as well as the indications for treatment in other conditions. In addition there needs to be vigilance for the development of side-effects related to this technique. For this reason it is important that treatments for other indications be conducted in the framework of research protocols.

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