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Regenerative medicine using dental pulp stem cells for liver diseases

Shogo Ohkoshi, Hajime Hara, Haruka Hirono, Kazuhiko Watanabe, Katsuhiko Hasegawa

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Abstract

Acute liver failure is a refractory disease and its prognosis, if not treated using liver transplantation, is extremely poor. It is a good candidate for regenerative medicine, where stem cell-based therapies play a central role. Mesenchymal stem cells (MSCs) are known to differentiate into multiple cell lineages including hepatocytes. Autologous cell transplant without any foreign gene induction is feasible using MSCs, thereby avoiding possible risks of tumorigenesis and immune rejection. Dental pulp also contains an MSC population that differentiates into hepatocytes. A point worthy of special mention is that dental pulp can be obtained from deciduous teeth during childhood and can be subsequently harvested when necessary after deposition in a tooth bank. MSCs have not only a regenerative capacity but also act in an anti-inflammatory manner *via* paracrine mechanisms. Promising efficacies and difficulties with the use of MSC derived from teeth are summarized in this review.

Key words: Dental pulp; Mesenchymal stem cell; Regenerative medicine; Liver disease; Tooth bank

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Core tip: Dental pulp contains a mesenchymal stem cell population that has a similar gene expression pattern to that of the bone marrow and differentiates into cells of multi-cellular lineages. There have been several reports showing hepatic differentiation of this stem cell population in the presence of specific growth factors in serum-free culture medium. Their self-renewal and high proliferative capacities verify their stem-cell character and suggest that they are a promising cell source of regenerative medicine for refractory liver diseases. Currently, these cells are in the stage of animal studies to prove the efficacy and safety of dental pulp stem cell-

based medicine for liver diseases.

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INTRODUCTION

The liver has a remarkable regenerative capacity in both physiological and pathological situations. However, this regenerative capacity is still insufficient to compensate for the functions of end-stage liver cirrhosis and fulminant hepatic failure, and prognosis of these diseases is extremely poor. Orthotopic liver transplantation is currently the only way to save patients in these critical situations; however, chronic donor shortage, post-operative severe complications, cost-effectiveness, and ethical issues always limit its application^[1].

There has always been a high expectancy that the remarkable regenerative capacities of stem cells will be used to treat intractable diseases and improve their prognosis. Currently, regenerative medicine using induced pluripotent stem cells (iPSCs) is attracting the most clinical attention^[2]. The first clinical trial of a retina pigment epithelium cell transplant derived from iPSCs for the treatment of age-related macular degeneration was conducted in Japan in 2014^[3]. In another study, Takebe *et al*^[4] succeeded in creating artificial liver buds using iPSC cells.

However, because iPSC cells do not exist in nature and are obtained artificially by inducing foreign genes or proteins, unexpected tumorigenesis and immunological rejections are always clinical concerns when using these cells. It has also been suggested that the induced genes might affect the expression of cellular genes^[5].

Mesenchymal stem cells (MSCs) are pluripotent cells that differentiate into variety of cell types. In particular, MSC from dental pulp (MSC-DP) has attracted clinical attention because they are easily obtained from extracted wisdom teeth or even from the deciduous teeth of children. This is in contrast to the collection of bone marrow MSCs for which a painful medical procedure is needed. MSC-DP have a marked proliferative capacity and can be passaged scores of times without losing their stem cell properties^[6]. Thus, this cellular resource is considered to be a promising source of cells for regenerative medicine that could be applicable to a variety of impaired organs, including diseased livers^[7]. In this review, recent experimental development of MSC-DP therapy for liver diseases is summarized.

MSCS AND THEIR APPLICATION TO REGENERATIVE MEDICINE

The recent developments in regenerative medicine

using stem cells have been outstanding. Application of autologous tissue stem cells to treat injured organs is the ideal method of regenerative medicine because, unlike the use of iPS cells, these methods do not require the induction of foreign genes or proteins, which possibly decreases the risk of tumorigenesis. Additionally, it does not involve critical ethical issues such as those encountered with embryonic stem cell (ES cell) therapies. Organ stem cells reside in almost all tissues and have the abilities of self-renewal and multi-lineage differentiation. They include hematopoietic stem cells (HSCs), MSC, neural stem cells, and skin and gut stem cells. These cells are relatively easily obtained by low-invasive procedures such as bone marrow aspiration, by using operative material or even by re-using discarded tissues such as umbilical cord or teeth. In particular, it is expected that the tooth bank will be used as a practical source of cells for regenerative medicine in the near future^[8,9].

MSCs have been most extensively studied using bone-marrow stem cells. Pittenger *et al*^[10] reported the multilineage potential of monolayer-cultured MSCs derived from bone marrow. MSCs exist in the stromal cells of bone marrow where they represent only 0.001%-0.1% of the total population of nucleated cells^[10,11]. They are adherent cells that show high proliferative potential in the presence of bFGF and hence, a homogeneous clone can be obtained by cell cloning^[12]. MSCs were shown to differentiate into multiple lineages such as neurons, muscle, skin cells, and hepatocytes. They are positive for CD44, CD73, CD90, CD105, CD271, and STRO-1 and negative for hematopoietic cell markers such as CD34 and CD45^[12].

Although hepatocytes have previously been considered to differentiate from endodermal cells, they have now been found to differentiate even from non-endodermal cells. Research involving the differentiation of MSCs into hepatocytes has mainly used MSCs from bone marrow. Lagasse *et al*^[13] transplanted HSCs into a model mouse of tyrosinemia and found that they engrafted in liver and improved of liver function. Krause *et al*^[14] showed that a single HSC clone not only reconstituted bone marrow but also differentiated into lung, skin, liver, and gut cells. Schwartz *et al*^[15] reported the culture of MSC derived from bone marrow in the presence of FGF-4 and HGF and showed that these MSCs developed the capacity to produce albumin and urea, which indicated the presence of progenitor cells of hepatocytes.

Subsequently, it was shown that such features were not limited to MSCs from bone marrow; MSCs from adipose tissue and placenta were also shown to differentiate into hepatocytes^[16,17].

LIVER REGENERATION STUDIES USING STEM CELLS

Terai *et al*^[18] administered bone marrow cells derived

from GFP-labelled mice to carbon tetrachloride-induced liver injury model mice and found that these bone-marrow cells engrafted in the injured liver, resulting in the absorption of fibrosis and the improvement of prognosis. Based on these experimental results, clinical trials of autologous bone marrow cells for end-stage liver cirrhosis patients started in November, 2011, in Japan^[19]. Many other clinical trials of regenerative treatment for end-stage liver cirrhosis using HSCs have also been reported. Pai *et al.*^[20] reported the improvement in the liver function of alcoholic cirrhotic patients who were administered CD34-positive cells that were induced by G-CSF treatment.

While general anesthesia is needed to obtain a sufficient number of bone marrow cells for treatment, MSCs can be expanded from a small volume of bone marrow fluid because of their high proliferative capacity under simple culture conditions. MSCs have also been applied to the treatment of ischemic heart disease, cerebral infarction, and neurological or autoimmune disorders *via* the production of growth factors and cytokines, which stimulate the repair of injured tissues^[21]. Several clinical trials using MSCs for decompensated liver cirrhosis have also been reported since 2007^[22]. However, not all of these clinical trials showed efficacy of this treatment^[23].

MSCS DERIVED FROM DENTAL PULP

Dental pulp is surrounded by dentin and is located in an enclosed space that connects with the external space through the apical foramen. Dental pulp has a strong capacity for repairing worn-down or carious teeth by producing dentin. Bone tissues are occasionally produced in the healing process of dental pulp. Dental pulp polyps are formed as granuloma tissues when squamous epithelium is formed that covers nerves that are exposed due to dental caries. These phenomena suggest that dental pulp has the capacity to develop into cells of multiple lineages, forming both bone and squamous epithelium.

Dental pulp is a mesenchymal tissue derived from dental papillae. Dental pulp cells have been reported to express bone markers similar to those expressed by osteoblasts^[24]. Gronthos *et al.*^[25] were the first to report the presence of MSC-DP. They showed that dental pulp cells from adult teeth became clonogenic and rapidly proliferated under culture conditions. The cells formed densely calcified nodules under osteo-inductive culture conditions and also formed dentin/pulp-like complexes when conjugated with hydroxyapatite/tricalcium phosphate, which revealed their stem cell characters. They further showed that MSC-DP also displayed a multi-lineage capacity, differentiating into adipocytes and neural cells, which seemed to be irrelevant to tooth function^[26]. The gene expression profiles of MSC-DP were shown to be similar to those of osteoblasts or

bone marrow stromal stem cells^[27].

Because MSC-DP are positive for STRO-1 and most STRO-1-positive MSC-DP are positive for pericyte-associated antigen, MSC-DP are considered to have originated from perivascular cell populations^[28]. Although the first MSCs were obtained from adult teeth, MSCs have also been derived from human exfoliated deciduous teeth (SHED), periodontal ligament^[29], apical papillae of immature permanent teeth^[30], or periapical cysts^[31]. In particular, SHED have a distinct capacity by virtue of higher proliferative potential than adult teeth with a multi-lineage differentiation capacity^[32]. SHED are easily applicable to a cell banking source such as that used for umbilical cord because of the low ethical hurdles and the fact that the concept of the re-use of discarded tissues is easily acceptable to the general public^[33]. Recent studies have shown that MSC-DP might induce immune regulatory mechanism of the host and have indicated the possibility of the application of MSC-DP to clinical practice^[34,35].

DIFFERENTIATION OF DP-MSC INTO HEPATOCYTES AND REGENERATIVE MEDICINE

The above information suggested that MSC-DP may be a promising cell resource for regenerative medicine for various organs. Ishkitiev *et al.*^[36] were the first to report that MSC-DP differentiated into hepatocyte-like cells. They cultured SHED in the presence of HGF, dexamethasone, and oncostatin, and found that they transformed into a hepatocyte-like shape and produced IGF-1 and albumin. They also identified the presence of urea in the culture medium, which suggested the possibility that the urea cycle was functioning in these cells. They purified CD117-positive cells from MSC-DP using magnetic cell sorting and succeeded in inducing hepatic differentiation of these cells in serum-free medium with a high efficacy^[37]. Since these cells still maintained stem cell markers such as embryonic (nanog), mesenchymal (CD44H), endodermal (nestin, CK19), ectodermal (p63), and mesodermal (SPARC, alkaline phosphatase, STRO-1) even after 70 passages, they may be applicable as a solid cell resource for regenerative medicine that can be obtained in sufficient cell numbers^[37]. The efficacy of MSC-DP in differentiating into hepatocytes was as high as that of bone marrow-MSC^[38]. When incubated with hydrogen sulphide, MSC-DP acquired more characteristic features of hepatocytes, showing a higher urea metabolism and glycogen synthesis^[39]. Hepatocytes that were differentiated from MSC-DP repopulated the cirrhotic livers of rats and were shown to improve liver function and survival of the animals^[7]. Yamaza *et al.*^[40] reported that transplanted SHED ameliorated liver dysfunction and improved inflammation and fibrosis in carbon tetrachloride - induced liver fibrosis model mice.

TOOTH BANK FOR REGENERATIVE MEDICINE

There is emerging interest in using MSC-DP as a clinical resource of cells for regenerative medicine for myocardial infarction^[41], rheumatoid arthritis^[42], diabetes mellitus^[43], Parkinsonism^[44], Alzheimer diseases^[45], and refractory muscle diseases^[46]. SHED derived from primary teeth are immature and have higher potential stem cell characteristics than adult-derived cells in terms of their proliferative capacity^[32]. The benefits of SHED cell banking are as follows:

Minimum immunological rejection since the cells are derived from an autologous source: Cell banking is possible at a very young age, long before illness manifests; the cells are obtained painlessly; low cost compared to umbilical cord cells; low ethical hurdles.

SHED are suitable for obtaining cells of multiple lineages such as cells of connective tissue, teeth, nerve, liver, and pancreas, while umbilical cord MSCs are suitable for obtaining HSCs.

A large number of SHED cells can be obtained because of their high proliferative capacity and cloning of cells derived from a single MSC clone is possible.

MSC-DP is covered by enamel tissue and has little exposure to external radiation, which is related to a lowered risk of carcinogenesis of the graft.

A tooth bank for the storage of MSC-DP from deciduous teeth has been established by public-private collaboration^[33]. However, many practical problems remain to be solved such as cost-benefit issues based on the balance of the risk of suffering diseases with the cost of long-term storage and harvest of cells, safety, and ethical concerns.

FUTURE DIRECTIONS

It has been reported that engrafted MSC do not actually transdifferentiate into specific cell lineages, but instead fuse with host cells using their plasticity^[47,48]. MSCs are less potent than ES cells. In addition, MSC-DP, similar to MSCs in general, not only contribute to tissue repair as an actual source of regeneration, but they also elaborate immunomodulatory or anti-inflammatory functions that may affect the local environment of transplanted tissues^[34,35,49]. There have been studies that showed that the conditioned medium of MSC cultures exerted immunomodulatory effects through paracrine mechanisms, that were mediated by extracellular vesicles such as exosomes produced by these cultured cells^[50-52]. Moreover, MSCs were reported to improve the levels of liver injury and attenuate fibrosis in animal models^[53,54]. These tissue repair effects of MSC-DP that occur through MSC-DP mediated paracrine mechanisms should be elucidated in parallel with studies to clarify the capacity of MSC-DP-derived hepatocytes as a substantial source of repopulating hepatocytes for fatal liver diseases.

Takebe *et al.*^[4] recently proposed the concept of

“organ buds” instead of organ and cell transplantation. They obtained a liver bud by co-culturing hepatocytes derived from iPS cells with MSCs and vascular endothelial cells. That study indicated the possibility that MSC might not be a central player in regenerative medicine, providing a substantial hepatic function, but might instead be a supporting player in the development of regenerating organ. Based on this concept, these researchers recently showed that MSCs contributed the formation of an organ bud by providing MSC-dependent cytoskeletal contraction force^[55].

These effects of MSC-DP on promotion of damaged-liver tissue repair through a paracrine mechanism or by an auxiliary force, should be elucidated in future studies.

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Tryptophan: A gut microbiota-derived metabolites regulating inflammation

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Crohn's disease and ulcerative colitis, are chronic intestinal disorders with an increased prevalence and incidence over the last decade in many different regions over the world. The etiology of IBD is still not well defined, but evidence suggest that it results from perturbation of the homeostasis between the intestinal microbiota and the mucosal immune system, with the involvement of both genetic and environmental factors. Genome wide association studies, which involve large-scale genome-wide screening of potential polymorphism, have identified several mutations associated with IBD. Among them, *Card9*, a gene encoding an adapter molecule involved in innate immune response to fungi (*via* type C-lectin sensing) through the activation of IL-22 signaling pathway, has been identified as one IBD susceptible genes. Dietary compounds, which represent a source of energy and metabolites for gut bacteria, are also appreciated to be important actors in the etiology of IBD, for example by altering gut microbiota composition and by regulating the generation of short chain fatty acids. A noteworthy study published in the June 2016 issue of *Nature Medicine* by Lamas and colleagues investigates the interaction between *Card9* and the gut microbiota in the generation of the microbiota-derived tryptophan metabolite. This study highlights the role of tryptophan in dampening intestinal inflammation in susceptible hosts.

Key words: Intestinal inflammation; Tryptophan; Microbiota

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Core tip: A noteworthy article published in *Nature Medicine* by Lamas and colleagues highlights the role of tryptophan, a microbiota-derived metabolite, in reducing inflammation in the gut. This commentary puts in perspective the main results from this study.

Abstract

Inflammatory bowel diseases (IBD), which comprise

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COMMENTARY ON HOT TOPICS

The human intestinal tract harbors a complex community including 100 trillion of microbes, referred as intestinal microbiota. This diverse microbial ecosystem provides benefits to the host, essentially through its role in energy metabolism and immunity. However, perturbations of gut microbiota (termed dysbiosis) is associated with several disorders, including inflammatory bowel disease (IBD) and metabolic syndrome (obesity-associated diseases)^[1]. IBD arise as a complex interaction between host genetic factors, mucosal immune system, intestinal dysbiosis, and environmental factors among which dietary compounds being increasingly appreciated in the onset of inflammatory related disorders. Unraveling the complex crosstalk between these factors arise as a challenge for the understanding and treatment of these disorders. A study published in the June 2016 issue of *Nature Medicine* by Lamas *et al.*^[2] made significant progress in this area by investigating how a gene predisposing to IBD (*Card9*, encoding the caspase recruitment domain-containing protein 9) leads to a colitogenic microbiota by impairing its ability to generate tryptophan-derived metabolite.

In their study, the authors reported that the deletion of *Card9* gene, a central component of the innate anti-fungal immune response, render mice more prone to chemically-induced colitis by dextran sulfate sodium (DSS)^[2]. This report strengthens previous studies conducted by others and identifying *CARD9* as a gene predisposing to IBD in humans^[3-5]. Lamas *et al.*^[2] also demonstrated that *Card9* knockout mice (*Card9*^{-/-}) display alteration of immune-related signaling pathways in the colon, with a strong decrease in interleukin-22 (IL-22) production. The authors evidenced a shift in the bacterial communities and alterations in the composition of the fungal microbiota in *Card9*^{-/-} mice. Complex inter-kingdom relationships exist in the gut microbiota, suggesting a possible role of *CARD9* in shaping the bacterial and fungal communities and required to control fungi during colitis. To decipher the mechanism of such colitis susceptibility and the involvement of gut microbiota in the onset of colitis, the authors use a model of microbial transplantation to germ-free recipient animals, and showed that transfer of colitic-associated microbiota of *Card9*^{-/-} susceptible hosts were sufficient to transferred colitis susceptibility and IL-22 cytokine production impairment in germ-free wild type (*Card9* sufficient) recipients. Those data strengthen the essential role played by the intestinal microbiota, bacteria but also fungi, in triggering intestinal inflammation following *Card9* impairment^[2].

Further analysis revealed that the colitic-associated microbiota of *Card9*^{-/-} mice is characterized by the absence of bacteria metabolizing tryptophan (an essential amino acid, whose intake is through the diet) into indoles derivatives, such as *Lactobacillus reuteri* and *Allobaculum* sp. Indoles derivatives are ligands for the aryl hydrocarbon receptor (AHR) that can drives local production of IL-22 by innate lymphoid cells and T-cells^[6]. Importantly, the authors described that the treatment of *Card9*^{-/-} susceptible animals with an AHR agonist [(i.e., 6-Formylindolo(3,2-b) carbazole named FICZ)] was sufficient to restore a normal level of IL-22 production and to protect mice from DSS-induced colitis. Previous studies focusing on the amino acid tryptophan demonstrated that mice fed with a low-tryptophan diet became susceptible to chemically induced inflammation^[7] and, conversely, mice or piglets fed with a tryptophan supplemented diet have a reduced inflammation and a decreased severity of DSS-induced colitis^[8,9].

As a therapeutic strategy, the authors next postulated that altering the intestinal microbiota in genetically susceptible host so as to increase its ability to generate AHR ligands could protect from intestinal inflammation. Thus, the authors demonstrated that supplementation with three commensal *Lactobacillus* strains with high tryptophan-metabolic activities was sufficient to restore intestinal IL-22 production and to reverse the colitis susceptibility observed in susceptible *Card9*^{-/-} mice. While previous studies have highlighted how diet can affect the microbiota in a detrimental way, such as the consumption of milk-fat-derived diet that lead to a bloom of pathobiont (i.e., *Bilophila wadsworthia*) and colitis in *IL10*^{-/-} mice^[10]; the study from Lamas *et al.*^[2] is a good example of the positive interplay between diet and the intestinal microbiota leading to the generation of microbial metabolites that play a central role in the protection against intestinal inflammation.

Finally, in their study, Lamas *et al.*^[2] further corroborated the results obtained in mice with the analysis of samples from IBD patients, and demonstrated that such patients have a reduced fecal AHR activity and fecal levels of tryptophan. The authors showed that these reductions correlate with *CARD9* polymorphism. These important findings consolidate the prominent role of dietary components and microbial-generated metabolites in mediating inflammation-related disorders. Tryptophan appears to be an important amino acid in IBD patients since they have lower levels of serum and fecal tryptophan compared to healthy subjects^[2,11]. In light of the close relationship occurring between the intestinal microbiota and dietary intake, such data further highlight the need of controlling both macro- and micro-nutrients consumption in IBD patients with genetic predisposition.

In the same issue of *Nature Medicine*, an additional study by Rothhammer *et al.*^[12] also expand the substantial effect of tryptophan in regulating inflammation, by focusing their study on the central nervous system

(CNS), and providing evidence on the significant role of the bidirectional communication between the gut microbiota and the brain. The authors found that mice fed with a tryptophan-deficient diet have exacerbated CNS inflammation, corroborating the results from Lamas *et al.*^[2]. These two reports support a potential probiotic strategy, wherein tryptophan-catabolizing *Lactobacillus* strains able to enhance AHR activity that can further beneficially impact the immune system through IL-22 production. Further exploration of possible manipulations of the gut microbiota through dietary modulations by a tryptophan-enriched diet or by re-shaping the microbiota *via* targeting specific populations of bacteria, for example by favoring the tryptophan-producing bacteria or by reducing its pro-inflammatory potential, will provide novel insights into the development of individual targeted approaches that can be harnessed to prevent and/or treat IBD patients.

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Pathogenic mechanisms of pancreatitis

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number of factors including pancreatic duct obstruction, alcoholism, and mutation in the cationic trypsinogen gene. Pancreatitis is represented as acute pancreatitis with acute inflammatory responses and; chronic pancreatitis characterized by marked stroma formation with a high number of infiltrating granulocytes (such as neutrophils, eosinophils), monocytes, macrophages and pancreatic stellate cells (PSCs). These inflammatory cells are known to play a central role in initiating and promoting inflammation including pancreatic fibrosis, *i.e.*, a major risk factor for pancreatic cancer. A number of inflammatory cytokines are known to involve in promoting pancreatic pathogenesis that lead pancreatic fibrosis. Pancreatic fibrosis is a dynamic phenomenon that requires an intricate network of several autocrine and paracrine signaling pathways. In this review, we have provided the details of various cytokines and molecular mechanistic pathways (*i.e.*, Transforming growth factor- β /SMAD, mitogen-activated protein kinases, Rho kinase, Janus kinase/signal transducers and activators, and phosphatidylinositol 3 kinase) that have a critical role in the activation of PSCs to promote chronic pancreatitis and trigger the phenomenon of pancreatic fibrogenesis. In this review of literature, we discuss the involvement of several pro-inflammatory and anti-inflammatory cytokines, such as in interleukin (IL)-1, IL-1 β , IL-6, IL-8 IL-10, IL-18, IL-33 and tumor necrosis factor- α , in the pathogenesis of disease. Our review also highlights the significance of several experimental animal models that have an important role in dissecting the mechanistic pathways operating in the development of chronic pancreatitis, including pancreatic fibrosis. Additionally, we provided several intermediary molecules that are involved in major signaling pathways that might provide target molecules for future therapeutic treatment strategies for pancreatic pathogenesis.

Key words: Pancreatitis; Pancreatic stellate cells; Transforming growth factor- β /SMAD; Janus kinase/signal transducers and activators; Mitogen-activated protein kinases

Abstract

Pancreatitis is inflammation of pancreas and caused by a

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Core tip: Pancreatitis is an acute or chronic inflammatory disease of the pancreas and characterized by destruction of acinar cells, which lead activation of several inflammatory cells like macrophages and granulocytes which secrete number of pro-inflammatory cytokines. These pro-inflammatory cytokines activate pancreatic stellate cells, *i.e.*, the key cells of pancreatic fibrosis. Various molecular signaling pathways (*i.e.*, transforming growth factor- β /SMAD, mitogen-activated protein kinases, Rho kinase, Janus kinase/signal transducers and activators, and phosphatidylinositol 3 kinase) are known to have critical role in the activation of pancreatic stellate cells in chronic pancreatitis and development of pancreatic fibrosis that lead to the pancreatic carcinoma.

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INTRODUCTION

Pancreatitis is a disease defined as acute or chronic inflammatory process of the pancreas characterized by premature activation of digestive enzymes within the pancreatic acinar cells and causing pancreatic auto-digestion^[1]. In pancreatitis, a local inflammatory process initiated by release of pro- and anti-inflammatory cytokines and chemokines recruits granulocytes, monocytes and lymphocytes^[2]. Annual incidence of acute pancreatitis varies from 13 to 45 per 100000 people in United States^[3], whereas chronic pancreatitis ranges from 4.4 to 11.9 per 100000 per year, with a higher occurrence in Japan as compared to the United States^[4-7]. Men are up to 1.5 times more likely to have chronic pancreatitis compared to women in the United States^[7]. In 2009, there were 19724 admissions for chronic pancreatitis in the United States, with associated annual hospitalization costs of \$172 million^[5,8]. However, the pathogenesis of chronic pancreatitis is not fully understood, but it is believed that repeated episode of acute damage lead chronic pancreatitis. Recurrent pancreatic injury leads to scarring and remodeling that promotes fibrosis as well as calcification, and these calcifications develop into stones found within the tissue or pancreatic duct^[5,9] (Figure 1). The main causes of pancreatitis are; obstruction in the main pancreatic duct, gallstones, alcohol misuse, smoking, hypercalcemia, hyperparathyroidism, drugs like valproate, thiazide toxicity, and genetic mutation^[10-12]. During pancreatic injury, atrophic acinar cells activate several inflammatory key players like macrophages and granulocytes which release a number of pro-inflammatory cytokines [*i.e.*, interleukin (IL)-1, IL-6, IL-8, IL-18, IL-33, and tumor necrosis factor (TNF)- α]. These pro-inflammatory

cytokines further activate pancreatic stellate cells (PSCs) to promote chronic pancreatitis^[13]. The detail of each cytokine involved in the pathogenesis of pancreatitis has been described independently.

PRO-INFLAMMATORY CYTOKINES

IL-1

Induction of IL-1 has been reported in acute pancreatitis and numerous reports implicated the role of IL-1, and IL-1 receptor (IL-1R) in the pancreatic pathogenesis^[14-18]. Interestingly, it has been shown that IL-1R gene-deficient mice or treatment with IL-1 receptor antagonist (rhIL-1Ra) attenuates cerulein-induced chronic pancreatitis in mice^[16]. IL-1 converting enzyme (ICE) is responsible for the secretion of IL-1 β from pro-IL-1 β and experimental pancreatitis was significantly attenuated by pre-treatment with an ICE inactivator (VE-13045), resulting in reduced histological grading of pancreatitis and mortality. These findings were further supported by using ICE-knock out mice or intraperitoneal (i.p.) injection of ICE-inhibitor^[19]. Additionally, IL-1 β is also believed to play a role in the pathogenesis of pancreatitis. An elevated serum level of IL-1 β has been associated with the development of acute pancreatitis^[20]. Recently, Xu *et al.*^[20] have revealed that IL-1 β can induce trypsin activation and decreases the cellular viability of pancreatic acinar cells. These effects depend on impaired autophagy *via* intracellular calcium changes. Ca²⁺ signaling may be a promising therapeutic target for the treatment of pancreatitis^[20].

IL-6

IL-6 is a very important pro-inflammatory cytokine involved in inflammation and immune responses^[21]. An important role of IL-6 has been shown in the development of acute and chronic pancreatitis as well as in pancreatic cancer. IL-6 mediates its action *via* gp130 protein and leads activation of Janus kinase/signal transducers and activators (JAK/STAT) signaling pathway^[21]. Reported data have revealed that patients with pancreatitis indicated high serum levels of IL-6 as compare to healthy individuals^[22,23]. *In vitro* studies have shown enhanced secretion of IL-6 from human pancreatic peri-acinar myofibroblast cells in the presence of several inflammatory mediators (*i.e.*, TNF- α , IL-17, IL-1 β) and growth factors (*i.e.*, fibroblast growth factor-2) and this data further supports the crucial role of IL-6 in the pathogenesis of acute pancreatitis^[24,25]. Interestingly, neutralization of IL-6 by anti-IL-6 antibody therapy revealed suppression of STAT-3 activation in pancreatic acinar cells and consequently reduces the severity of acute pancreatitis^[26]. The abnormal expression and deregulation of IL-6 in pancreatitis suggested that IL-6 serves as a valuable early marker for pancreatitis.

IL-8

IL-8, known as chemokine (C-X-C motif) ligand 8 or

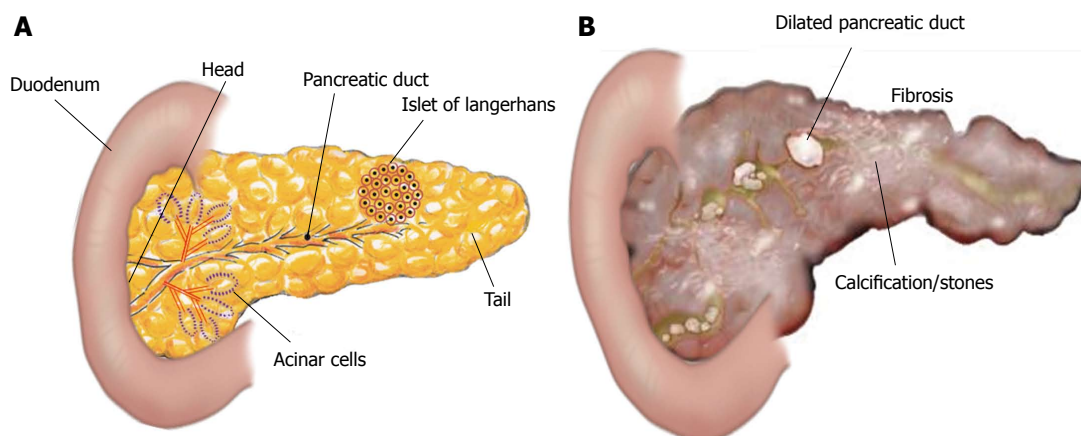


Figure 1 Structure of pancreas. A: The pancreas is a leaf-like structure and has two types of cells: Exocrine cells, that include acinar pancreatic duct cells, and endocrine cells, that include islets of Langerhans; B: The inflammatory process in the pancreas promotes fibrosis (scarring of tissue), calcifications or stones, and dilated pancreatic duct.

CXCL8, acts as a potent chemo-attractor of neutrophils and affects neutrophil function during onset of inflammatory responses by regulating the trafficking of various types of leukocytes through interaction with transmembrane receptors. IL-8 is produced by several types of cells such as monocytes/macrophages and epithelial cells^[27,28]. Systemic complications of acute pancreatitis are associated with higher levels of IL-8^[29-31]. Induction of IL-8 was also reported in a patient with aggravation of pancreatitis which suggests that IL-8 takes part in the pathogenesis of pancreatitis^[32]. Severity of acute pancreatitis is associated with polymorphisms of the *IL-8* gene^[33]. However, the mechanism of IL-8 mediated severity of acute pancreatitis is not yet well understood and requires further study in this area.

IL-18

Induction of IL-18 is now identified in a number of disorders, such as autoimmunity^[34], cutaneous^[35] and allergen-induced allergic responses^[36]. IL-18 is a member of IL-1 family cytokine and implicated in numerous aspects of the innate and adaptive immune system, with some analogy to IL-1 β ^[37]. Evidences indicate that IL-18 is induced in the blood of acute^[38] and chronic pancreatitis patients^[39,40]. Furthermore, higher serum level of IL-18 was also reported during mild and severe forms of acute pancreatitis compared to healthy controls^[41]. Additionally, the induced IL-18 level was also reported in taurocholic acid and endotoxin-induced acute pancreatitis in rat^[42]. Interestingly, IL-18 along with IL-12 induces severe acute pancreatitis in obese mice^[43]. Notably, it is also reported that the IL-18 has an important role in the progression of disease from acute to chronic stages^[40]. Overall, IL-18 seems to be released early during the course of acute pancreatitis and may act as a key immunomodulator of the inflammatory response in severe pancreatitis and associated fibrosis. However, the mechanistic pathway of IL-18-induced chronic pancreatic pathogenesis is yet not understood.

IL-33

IL-33, a new member of the IL-1 superfamily of cytokine, binds to a complex of the ST2L/IL1 receptor accessory protein (IL1RAcP), which mediates its function^[44]. Several investigations suggest a crucial role of IL-33 in the pathogenesis of chronic pancreatitis and possibly pancreatic cancer^[45,46]. IL-33 was found to activate acinar cell pro-inflammatory pathways and to exacerbate acute pancreatic inflammation in mice^[47]. However, the activated PSCs express IL-33 in the nucleus and regulate the platelet-derived growth factor (PDGF)-induced proliferation in PSCs^[48]. IL-33 also acts as a pro-inflammatory cytokine and modulates its receptor gene expression in Colo357 cells, *i.e.*, human pancreatic carcinoma cells^[45]. IL-33 and its receptor complex (ST2L and IL1RAcP) constitute a novel signaling system; therefore, this pathway may be important in promoting acute and chronic pancreatitis. Additionally, a role for IL-33 in the stimulation, proliferation and migration of pancreatic myofibroblasts is also reported^[46].

TNF- α

TNF- α is a pleiotropic cytokine and acts as a central regulator of inflammation^[49,50]. It is mainly secreted by monocytes and macrophages but is also released by pancreatic acinar cells after an inflammatory trigger^[51-54]. A number of studies have revealed TNF- α plays an essential role in the pathogenesis of pancreatitis and contributes inflammatory responses to disease pathogenesis^[51-53,55]. An *in vitro*-based study indicates cultured pancreatic acinar cells are able to produce, release, and respond to TNF- α ^[56], leading to the activation of nuclear factor-kappa B (NF κ B); interestingly, inhibition of NF κ B activity decreases the inflammatory response during experimental pancreatitis^[57,58]. Serum levels of TNF- α have not been considered to be a good indicator of disease severity because the liver is able

to rapidly clear TNF- α before it reaches the general circulation; therefore, it is often difficult to detect TNF- α in the serum of acute pancreatitis patients^[59]. One study indicates that TNF- α levels were higher in acute pancreatitis as compared to the chronic form of the disease, but its concentration did not correlate with the severity of disease^[60]. In contrast to this, a recent study has shown levels of TNF- α are also increased in patients with chronic pancreatitis and the concentration of TNF- α coordinately increases in advanced chronic pancreatitis^[61]. Furthermore, TNF- α mediates its effect by two surface receptors, TNF- α receptor 1 (TNFR1), or p55, and TNFR2, or p75, and both receptors are expressed in the pancreas^[54,62]. Interestingly, genetic deletion of TNFR1 prevents the activity of TNF- α and revealed beneficial effects on symptom severity and mortality in cerulein-induced pancreatitis^[63].

ANTI-INFLAMMATORY CYTOKINES

IL-10

IL-10 is produced by a number of activated immune cells like monocytes/macrophages, Treg, and Th1 cells^[64,65]. IL-10 gene deficient mice showed more inflammatory responses and lung injury during acute pancreatitis and chronic pancreatitis^[66,67]. Pre-treatment of IL-10 agonist (*i.e.*, IT 9302) was found to reduce lung injury and mortality in a rabbit pancreatitis model^[68]. Plasma IL-10 level was found to correlate with the severity of pancreatitis and could be used as a marker for severity prediction^[22,69]. Initial studies based on several rodent models of acute pancreatitis revealed a protective role of IL-10 by reducing the production of inflammatory cytokines from macrophages and also diminished the level of serum amylase, serum lipase, edema, necrosis and hemorrhage^[70-72]. However, recombinant IL-10 treatment in human pancreatitis has given mixed responses^[73]. In summary, IL-10 holds the promise of a global attenuation of the cytokine response, and more work is needed to establish its beneficial use in pancreatitis.

GRANULOCYTES INFILTRATION IS CRITICAL IN THE PATHOGENESIS OF CHRONIC PANCREATITIS

Granulocytes infiltration in the pancreas is implicated in the initiation and progression of pancreatic inflammation. The major granulocytes identified in acute and chronic pancreatitis patients are neutrophils and eosinophils. Neutrophils play a crucial role in acute inflammatory pancreatitis, are attracted to the site of injury by the help of chemokines such as CXCL8 in humans as well as CXCL1 in mouse, and further regulate the immune responses. Neutrophils remain in the blood circulation and have a very short life of approximately 24 h^[74]. However, in an inflammatory condition they became activated and their lifespan is prolonged for

several days, during which they control inflammatory responses and activate several pro-inflammatory mediators^[75]. Trypsinogen activation is the key step for progression of pancreatitis; and a report suggested that initial trypsinogen activation is not regulated by neutrophils, whereas later activation of trypsinogen during pancreatitis is dependent on neutrophils^[76]. In addition, several cases have been reported in the literature indicating the presence of increased number of eosinophils in patients with pancreatitis and termed this condition as "Eosinophilic Pancreatitis"^[77,78]. Eosinophilic pancreatitis is a rarely occurring disorder and reports indicate that eosinophilic pancreatitis is frequently diagnosed only after "false positive" pancreatic resection for suspected pancreatic tumor and mimic pancreatic neoplasm^[78,79]. The first report of peripheral blood eosinophilia in a patient with chronic relapsing pancreatitis with pleural effusion was published by Juniper^[80] in 1955 and thereafter, several evidences came in the literature^[81-84]. Tokoo *et al.*^[81] performed a study of 122 patients with chronic pancreatitis and found marked eosinophilia in approximately 21 cases (17.2%). All of the affected patients were males; no females were found affected. Endocrine pancreatic function was normal in the chronic pancreatitis patients with eosinophilia, whereas marked exocrine pancreatic dysfunction was observed in these patients. The eosinophilia of chronic pancreatitis has been frequently developed in association with severe damage to adjacent organs (pleural effusion, pericarditis, and ascites), as well as an association with pancreatic pseudocyst. This finding suggests that there may be a close correlation between marked eosinophilia and severe tissue injury during acute exacerbations of chronic pancreatitis^[81]. Another study revealed 28 cases (15.6%) of chronic pancreatitis with eosinophilia among 180 chronic pancreatitis patients and the ratio of male to female patients was 8.3:1. The occurrence of eosinophilia during the course of chronic pancreatitis might be responsible for the progression of pancreatic inflammation and fibrosis^[82]. Additionally, reports indicate that peripheral eosinophilia, allergic disorders, and pancreatic eosinophil infiltration have been associated with autoimmune pancreatitis^[83,84]. Diagnosis and treatment of eosinophilic pancreatitis is important as it promotes pancreatic fibrosis and neoplasm.

EXPERIMENTAL TOOLS TO DISSECT THE MECHANISM THAT PROMOTES PANCREATITIS

Pathogenesis of pancreatitis is essentially understood by using experimental animal models, because of the anatomical location of the pancreas and the difficulty in procuring human tissue at different stages of the inflammatory process. Several animal models are reported to understand the pathogenesis of pancreatitis, which enable us to develop more effective treatment therapies to improve the quality of life of patients suffering

from pancreatitis-associated complications. In brief, we summarize some experimental models used for understanding the disease initiation and progression.

Cerulein-induced pancreatitis model

The most widely used acute and chronic pancreatitis model, the cerulein-induced model is a highly reproducible and economical model in rats and mice^[85-87]. Acute pancreatitis can be induced by intraperitoneal (*i.p.*) injection of cerulein (5 µg/kg per hour in rats and 50 µg/kg in mice) several times at hourly intervals, and repeated doses of cerulein can induce chronic pancreatitis^[88,89]. Cerulein is an analog of cholecystokinin^[90] and induces the secretion of digestive pancreatic enzymes from pancreatic acinar cells like amylase and lipase. Cerulein treatment further causes infiltration of inflammatory cells within the pancreas, pancreatic edema, and acinar cells vacuolization that are comparable to acute pancreatitis in humans. Cerulein-induced pancreatitis model has been considered as a representative model of mild acute pancreatitis of human.

L-arginine-induced pancreatitis model

Another experimental and reproducible pancreatitis model is L-arginine-induced model. This model is also widely used to study the pathophysiology of acute necrotizing pancreatitis to produce acinar cells necrosis. Initially, Mizunuma *et al.*^[91] and Tani *et al.*^[92] have demonstrated *i.p.* administration of excessive doses of L-arginine (500 mg/100 g body weight) in rat caused damage of pancreatic acinar cells. A single *i.p.* dose of 500 mg/100 g revealed necrosis in 70%-80% of acinar cells within 3 d^[91,92]. Since, these observations, the L-arginine-induced acute pancreatitis rat model has been used by several investigators^[93,94].

Bile salt-induced pancreatitis model

The first experimental biliary acute pancreatitis model was established by Bernard in 1856 *via* retrograde injection of bile and olive oil into the pancreas of a canine^[95]. Since then, various bile salts such as sodium chenodeoxycholate^[96], sodium taurocholate, sodium glycodeoxycholic acid^[97], sodium-taurodeoxycholate and tauro lithocholic acid 3-sulphate have been reported to induce acute pancreatitis in different animal models. Among these bile salts, the taurine-conjugated bile salt sodium taurocholate was the most widely used and best characterized chemical for the induction of acute pancreatitis^[98]. Furthermore, a choline-deficient, ethionine-supplemented diet model is another established model to study the pathogenesis of acute and chronic pancreatitis^[99,100].

Pancreatic duct ligation model

In the rat model of pancreatitis, bile reflux was first implicated in the disease pathogenesis and termed as biliary pancreatitis^[101,102]. Biliary pancreatitis develops

from obstruction by gallstone or bile reflux into the pancreatic duct, which causes induction of acute pancreatitis. The rat model shows that due to high pancreatic duct pressure, pancreatic juice refluxes into the bile duct in the presence of ampullary orifice obstruction, resulting in pancreatic edema, inflammatory cell infiltration, increased amylase production^[103]. Chronic pancreatitis develops in these mice with time that includes atrophy, loss of acinar cells, and fibrosis^[103,104].

Alcohol-induced pancreatitis model

Alcohol is another accountable factor for the pathogenesis of pancreatitis, and it has been used to trigger chronic pancreatitis in animal models^[85,105,106]. Lieber and DeCarli have investigated the effects of ethanol on several organs by giving repeated feedings of ethanol as a part of the diet to rats and baboons^[107] and the animals developed fatty liver disease, alcoholic hepatitis, and later on cirrhosis. Undesirably, alcohol ingestion alone did not induce chronic pancreatitis despite long experimental durations. However, the combination of alcohol with various agents such as cerulein or lipopolysaccharide exacerbated pancreatitis and resulted in fibrosis^[108]. Activation of pancreatic stellate cells and fibrosis has been observed in the rat given isocaloric Lieber-DeCarli liquid diets along with alcohol for up to 10 wk and challenged with 1 or 3 repeated doses of lipopolysaccharide^[109]. Alcohol-induced pancreatic damage is thought to be mediated by its metabolites, which activates ROS to cause acinar cells injury and activate pancreatic stellate cells, leading to fibrosis^[110].

SNARE proteins mediating basolateral exocytosis in alcohol-induced pancreatitis

The important role of SNAREs [soluble NSF (N-ethylmaleimide-sensitive fusion proteins) attachment proteins receptors] mediating basolateral exocytosis in alcohol-induced pancreatic injury has been reported^[111]. SNARE proteins are of two types (1) t-SNAREs present on the target membrane, and (2) v-SNAREs, positioned on the membrane of vesicles. The t-SNAREs, syntaxin and synaptosome-associated proteins, together make a SNARE complex which binds to v-SNAREs and triggers the fusion of vesicle and target membranes. In the pancreas, this leads to release of zymogen granules into the ducts for transport to the duodenum for their activation^[112]. Additionally, a study indicates ethanol/cholecystokinin-evoked pancreatic acinar basolateral exocytosis has been mediated *via* protein kinase C alpha phosphorylation of Munc18c, which enables Syntaxin-4 to become receptive in forming a SNARE complex in the basolateral plasma membrane. The authors also considered this phenomenon as an operating mechanism contributing to alcoholic pancreatitis^[111]. Importantly, displacement of Munc18c from the pancreatic acinar basal membrane surface has been observed in tissue samples from a patient suffering from alcohol-induced chronic pancreatitis^[113].

CHRONIC PANCREATITIS LEADS FIBROSIS AND PANCREATIC CANCER

Chronic pancreatitis develops fibrosis and it is the common pathological characteristic feature and major risk factor for pancreatic cancer^[114]. Recent data has shown 48960 new cases of pancreatic cancer arise and 40560 deaths occur annually in the United States because of pancreatic cancer^[115]. Chronic pancreatitis is a long-standing inflammation of the pancreas that often leads to permanent damage of pancreas and serious complications, including pancreatic cancer. Chronic pancreatitis is characterized by marked stroma formation with an increased number of infiltrating macrophages and stellate cells, which are believed to play a central role in triggering inflammation and disease progression. The treatment of chronic pancreatitis and pancreatic cancer remains problematic as tissue becomes fibrotic due to injury that triggers several inflammatory, cellular as well as molecular signaling cascades that lead to formation and deposition of extra cellular matrix (ECM) at the site of injury. Several key cells are known to be involved in the process of fibrogenesis, such as inflammatory cells (e.g., macrophages and T cells), epithelial cells, fibrogenic effector cells, and endothelial cells. There are different types of effector cells in different organs, such as fibroblasts, myofibroblasts, and fibrocytes^[116]. Among these cells, fibroblasts and myofibroblasts are the key cells in fibrosis and are responsible for secretion of ECM^[117]. However, the function of fibrocytes is similar to the fibroblasts but to a lesser extent. Apart from this, macrophages have a more indirect contribution to fibrosis through their roles in chronic inflammation by producing a wide range of cytokines such as, transforming growth factor- β (TGF- β), PDGF, fibroblast growth factor 2 (FGF2) and insulin-like growth factor 1, all of them have pro-fibrotic effects on fibroblasts^[118,119]. If fibrogenic processes persist for long time, parenchymal scarring, cellular dysfunction and organ failure take place^[116]. Fibrosis is becoming a global problem and it can be of various types depending upon the tissue where it happened, such as cardiac, hepatic, renal, pulmonary, skin, liver and pancreatic fibrosis, etc. Fibrosis is an irreversible process and most of the drugs are not effective to treat fibrosis. Restriction of the progression of fibrogenesis might be a promising approach for the treatment of several fibrotic diseases.

Herein, our focus is on pancreatic fibrosis that happens during repeated injury to the pancreas. The normal pancreas has two major functions: (1) exocrine; and (2) endocrine. Exocrine pancreas comprises more than 95% of the pancreatic mass and consists of two types of pancreatic cells: (1) acinar cells, which produce digestive enzymes; and (2) ductal cells lining pancreatic ducts, which secrete a watery fluid to transport the digestive enzymes into the intestine. Endocrine pancreas mainly consists of the islets of Langerhans, which secrete insulin and other hormones^[5,120] (Figure 1).

Development of fibrosis is a dynamic phenomenon that requires an intricate network of several autocrine and paracrine signaling pathways^[116]. In this process, ECM formation takes place in the interstitial spaces and in areas where the exocrine compartment, mainly acinar cells are damaged^[121,122]. Pancreatic injury activates acinar cells, macrophages and neutrophils which induces pro-inflammatory cytokines (IL-1, IL-6, and IL-8), chemokines (monocyte chemoattractant protein-1, macrophage inflammatory protein-1) and growth factors, which further activate quiescent PSCs^[2]. The available facts suggest that these activated PSCs are the main cells in the development of fibrosis during chronic pancreatitis *via* secretion of TGF- β , FGF and COX-2 which leads to synthesis of ECM^[123,124]. A schematic mechanistic pathway involved in the progression of chronic pancreatitis is shown below in Figure 2.

Furthermore, activated PSCs have the ability to synthesize and secrete several matrix proteins, matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases, thus indicating that PSCs have dual functions to regulate the physiology of the exocrine pancreas, *i.e.*, they can synthesize as well as degrade the extracellular matrix^[125,126]. This indicates that PSCs have the ability to make a balance between fibrogenesis and matrix degradation to regulate the health of pancreatic tissue; that is, conservation of normal architecture or development of progressive fibrosis. Fibrosis is a complex process and the mechanism of pancreatic fibrosis is still not well understood. Due to pancreatic fibrosis, a number of therapies in pancreatic cancer have failed. In our standing, for proposing or designing any therapeutic strategy for chronic pancreatitis or pancreatic cancer, the mechanism of fibrosis development in the pancreas is important. Herein, we provide a summary of various molecular signaling pathways [*i.e.*, TGF- β /SMAD, mitogen-activated protein kinase (MAPK), Rho kinase, JAK/STAT, and phosphatidylinositol 3 kinase (PI3K)] that have been shown to play a critical role in the activation of PSCs during chronic pancreatitis and trigger the phenomenon of fibrogenesis in pancreas (Figure 3).

TGF- β 1/SMAD PATHWAY

TGF- β is a multipotent cytokine and exists in three isoforms (TGF- β 1, TGF- β 2 and TGF- β 3) in mammals and plays an integral role in regulating immune responses, cell growth, cell differentiation and apoptosis^[127,128]. TGF- β mediates its downstream signaling by binding to its specific receptors and triggers the activation of several SMAD proteins, which acts as chief transducers of the signal from the receptors to the nucleus. The receptor-regulated SMADs (R-SMADs), SMAD-2 and SMAD-3, are directly phosphorylated by the TGF- β 1 receptor and make a complex with the common mediator SMAD (*i.e.*, co-SMAD; SMAD-4) that

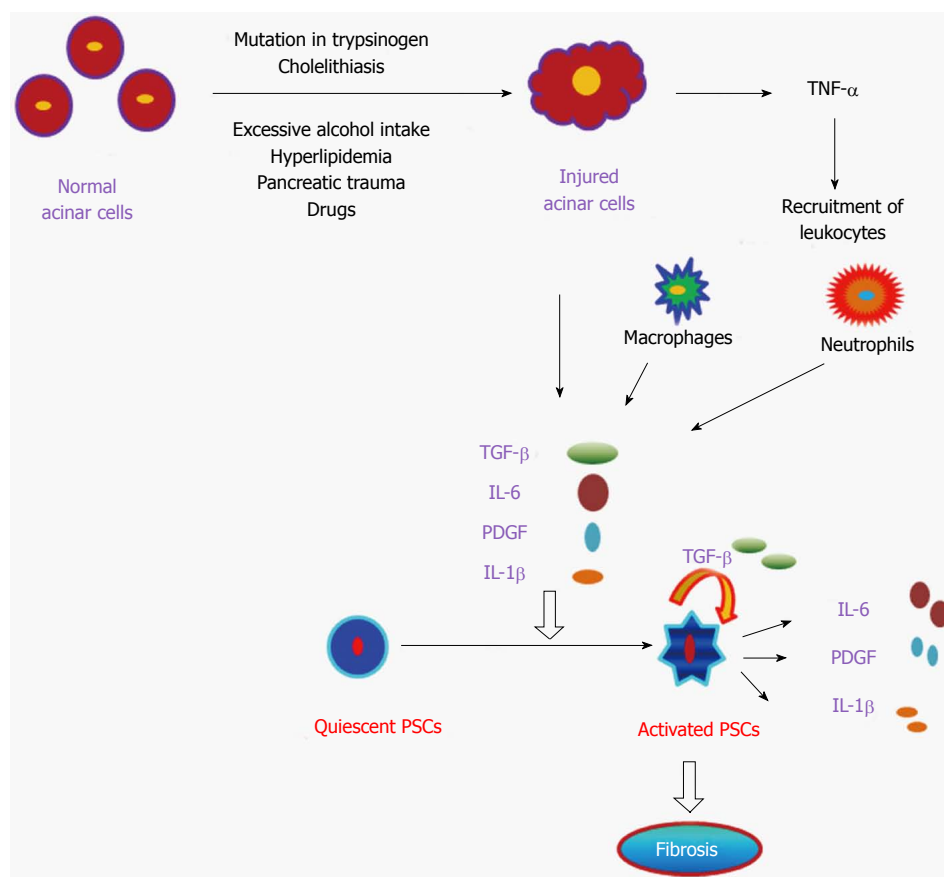


Figure 2 Pathogenesis of pancreatitis. Diagrammatic representation of the onset of pancreatitis by damaged pancreatic acinar cells which in turn activates quiescent pancreatic stellate cells (PSCs) to become activated PSCs and promote subsequent fibrosis of pancreas. TNF- α : Tumor necrosis factor- α ; TGF- β : Transforming growth factor- β ; PDGF: Platelet-derived growth factor; IL: Interleukin.

translocate into the nucleus and activates the transcription of target genes^[127,129,130]. Earlier studies have confirmed the involvement of TGF- β in the pathogenesis of acute pancreatitis, chronic pancreatitis and development of fibrosis^[131-136]. PSCs play a key role in triggering pancreatic fibrosis and interestingly TGF- β was found to regulate activation and proliferation of PSCs in an autocrine manner *via* involvement of SMAD-2, SMAD-3 and ERK pathways^[137,138]. Amelioration of pancreatic fibrosis in cerulein-treated mice was observed with defective TGF- β signaling by over-expressing a dominant-negative mutant form of TGF- β type 2 receptor (pS2-dnR II) only in the pancreas under control of pS2/TFF1 promoter^[139]. Subsequent study has revealed suppression of TGF- β signaling halts cerulein-induced pancreatitis^[140]. These studies indicate a functional TGF- β signaling pathway might be required for cerulein to induce acute pancreatitis in these mice^[139,140]. In contrast, deactivation of TGF- β signaling induces autoimmune pancreatitis in mice, indicating the important role of TGF- β either in maintaining immune homeostasis and suppressing autoimmunity or in preserving the integrity of pancreatic acinar cells^[141]. Transgenic mice with an S100A4/fibroblast-specific protein 1 Cre-mediated conditional knockout of TGF- β type 2 receptor spontaneously developed autoimmune pancreatitis in 6 wk. This indicates autoimmune pancrea-

titis resulted from loss of TGF- β signaling in S100A4-positive dendritic cells^[142].

Plenty of evidence suggests the involvement of TGF- β in pancreatic fibrosis, however TGF- α was also found to increase the proliferation as well as migration of PSCs *via* up-regulation of MMP-1, which might contribute to the pathogenesis of chronic pancreatitis^[143]. A recent report has shown loss of SMAD-4 synergizes with TGF- α over-expression in promoting pancreatic metaplasia, PanIN development, and fibrosis^[144]. Furthermore, a higher level of TGF- β 1 during pancreatic inflammation triggers the deregulation of the microRNA-217-SIRT1 pathway and then promotes EMT and subsequent fibrosis in the pancreas^[145]. Although, TGF- β still remains elusive in terms of our understanding of its multifunctional modes of action and TGF- β also activates SMAD-independent signaling pathways including MAPK pathways and phosphoinositide (PI) 3-kinase^[129,146,147] but the detailed mechanisms are not well understood.

MAPK

MAPK are of three types, ERK, JNK, and p-38, and play an important role in a variety of cellular processes, including cell proliferation, cell survival, apoptosis, and cytokine production^[148]. In alcohol-induced pancreatic injury, ethanol and its metabolite acetaldehyde were

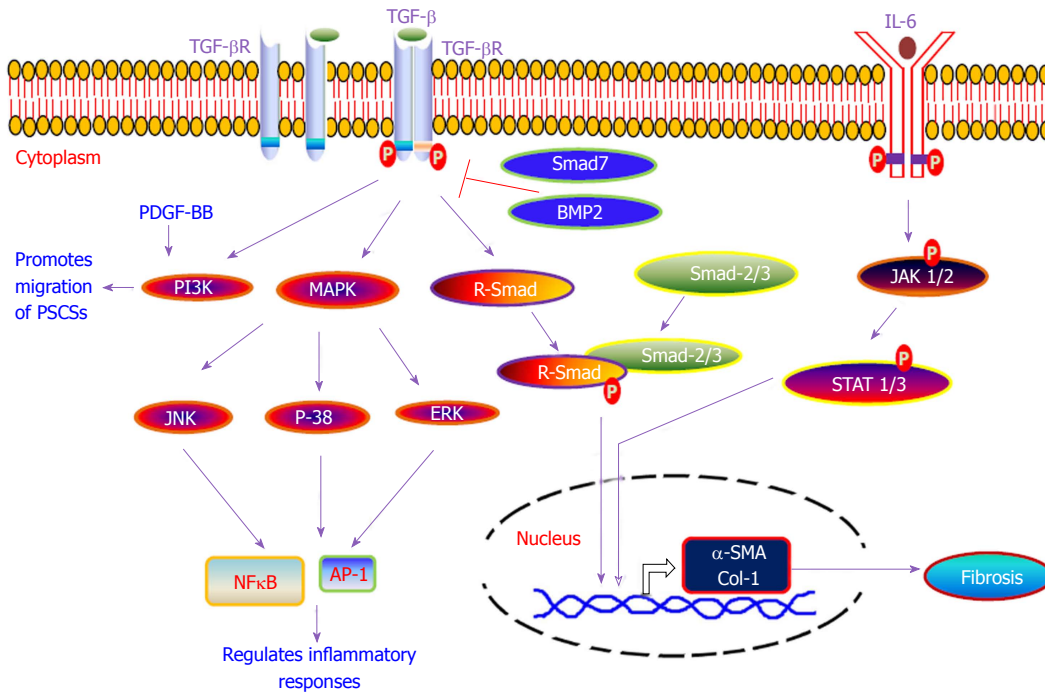


Figure 3 Various signaling pathways involved in the development of pancreatic fibrosis. Diagrammatic representation of various molecular signaling pathways which are involved in the development of pancreatic fibrosis. TGF- β : Transforming growth factor- β ; PDGF: Platelet-derived growth factor; IL: Interleukin; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3 kinase; AP-1: Activator protein-1; NF κ B: Nuclear factor kappa B.

found to induce activator protein-1 (AP-1) and MAPK signaling in PSCs^[149,150]. Furthermore, CX3CL1 is a chemokine that serves as an adhesion molecule as well as a migration factor, and was elevated in patients with alcoholic chronic pancreatitis^[151]. A recent report indicates ethanol induces CX3CL1 release *via* ERK activation in PSCs^[152]. However, H₂O₂ induces oxidative stress, AP-1, MAP-kinase pathway and expression of α (I) procollagen in PSCs^[153]. Apart from this, PDGF induces rapid activation of Raf-1, ERK 1/2, and AP-1 protein and further indicates a correlation between ERK activity and PSC activation^[154]. Furthermore, the involvement of protease-activated receptor-2 (PAR-2) was also found in the pathogenesis of pancreatitis and PAR-2 agonists increased collagen synthesis *via* activation of JNK and p-38 MAP kinase pathways in PSCs, suggesting the role of PAR-2 during induction of pancreatic fibrosis^[155]. PD98059 is an inhibitor of MAP/ERK kinase-1 (MEK-1) pathway and was able to protect against cerulein-induced acute pancreatitis in mice^[156]. Apart from this, angiotensin II-treated PSCs start proliferation and increase DNA synthesis *via* an epidermal growth factor receptor transactivation-ERK activation pathway, indicating the possible role of angiotensin II in development of pancreatic fibrosis^[157,158]. Taken together, these studies have broadened our knowledge to understand the role of the MAP kinase signaling pathway in the development of pancreatitis-associated fibrosis, but still the existence of several molecular signaling pathways which may cross-talk to each other have an important role in the development of fibrosis and need to be explored further.

RHO KINASE PATHWAY

In chronic pancreatitis, activation of PSCs and induced stress fiber formation suggest the reorganization of cytoskeletal proteins is involved in this disease process^[159]. The Rho family proteins RhoA, Rac and Cdc42 are considered the core molecules that induce stress fiber formation and regulate cellular adherence by remodeling of the cytoskeleton in response to external signals^[160,161]. Further, the inhibition of Rho A signaling diminished the endothelial hyper-permeability that was induced by sera from severe acute pancreatitis patients with lung injury *via* inhibiting F-actin aggregates^[162]. Inhibitors of Rho kinase such as Y-27632 and HA-1077 (fasudil) block activity of PSCs, *via* reducing α -SMA, proliferation, chemotaxis, and type I collagen production in culture-activated PSCs^[163]. During cerulein-induced pancreatitis in mice, Y-27632 caused induction of serum amylase levels, higher interstitial edema and vacuolization at 12-18 h after the first injection of cerulein. Y-27632 in turn inhibited the recovery of protein expression of ROCK-II at 18 h after the first cerulein injection. These results indicate that RhoA and ROCK-II accumulate in normal CCK-stimulated pancreatic enzyme secretion and prevent cerulein-induced acute pancreatitis^[164]. Rho-kinase signaling was found to regulate trypsinogen activation and its release from the pancreatic acinar cells during acute pancreatitis and subsequent CXC chemokine formation, neutrophil infiltration and tissue injury^[165]. Thus, these results indicate that Rho-kinase may serve as a novel molecular target for future treatment of acute pancreatitis, but there is need for

vast effort to understand the Rho-Kinase signaling in pancreatitis. This area opens up a new avenue for future research.

JAK/STAT SIGNALING PATHWAY

The JAK/STAT signaling pathway regulates several cellular functions such as cell proliferation, differentiation, and inflammatory responses^[166-168]. IL-6 is a well-known pro-inflammatory cytokine and mediates its action *via* JAK/STAT signaling pathway^[21] and plays a crucial role in the progression of pancreatitis. Various reports have indicated higher serum levels of IL-6 in patients with pancreatitis as compared to healthy individuals^[22,23]. Furthermore, an *in-vitro* study also indicates induced secretion of IL-6 from the human pancreatic periacinar myofibroblast cells under the influence of several inflammatory mediators, such as TNF- α , IL-17, IL-1 β and FGF-2; this data further indicates the crucial role of IL-6 in the pathogenesis of acute pancreatitis^[24,25]. Interestingly, blockade of IL-6 using anti-IL-6 antibody suppresses STAT-3 activation in the pancreatic acinar cells and consequently diminishes the severity of acute pancreatitis by induction of pancreatic acinar cell apoptosis^[26]. Apart from this, another report suggests that PDGF induces the proliferation of PSCs^[169] by activating the JAK-2/STAT-3 pathway^[155]. The inhibition of JAK-1/STAT-1 improves the severity of cerulein-stimulated pancreatic injury by inhibiting the activation of NF κ B, and this indicates that activation of JAK-1/STAT-1 is involved in the early events of pancreatic injury^[170]. Still, a better understanding of the JAK/STAT signaling pathway is required to know its role in the proliferation of PSCs and progression of fibrosis in chronic pancreatitis.

PI3K-AKT PATHWAY

PI3K-Akt is a major intracellular signaling pathway that belongs to a family of lipid and protein kinases. When growth factors bind to membrane bound receptor tyrosine kinase, it activates PI3K and its downstream regulators Akt and mTOR and regulates several aspects such as cell growth, survival, apoptosis and inflammation^[171-173]. Earlier, it has been shown the PI3K pathway inhibitor wortmannin reduces the intra pancreatic activation of trypsinogen in acinar cells^[174] and decreases inflammatory cytokines in severe acute pancreatitis in rats^[175]. These reports suggest involvement of the PI3K pathway in the pathogenesis of acute pancreatitis. PI3K γ is an isoform of PI3K known to regulate pathologic responses of the pancreatic acinar cells during pancreatitis^[176]. The role of PI3K γ was studied in two different models of acute pancreatitis, cerulein and choline-deficient/ethionine-supplemented diet. Mice lacking the *PI3K γ* gene are protected from acinar cell injury/necrosis and show reduced severity of acute pancreatitis, indicating PI3K inhibitors may provide a possible therapy for acute pancreatitis^[172,177].

CLINICAL CHARACTERISTICS AND DIAGNOSIS OF PANCREATITIS

The major clinical characteristics of pancreatitis are abdominal pain localized to the upper-to-middle abdomen, abdominal distension, nausea, fever, flank pain, vomiting, back pain, jaundice, hematemesis, melena diarrhea with foul-smelling, oily bowel movements and weight loss^[178]. Abdominal pain is the most common symptom found in 50% to 80% of cases, and it is the major cause for hospitalizations of patients related to pancreatitis. Although the pancreatic pain is low in the abdomen, following food intake it worsens and becomes localized to the epigastric area^[179]. Ammann *et al.*^[180] have identified two types of pancreatic pain (type A and type B) on the basis of natural history of alcoholic chronic pancreatitis. In type A pain, there are short (< 10 d) episodes of acute pain with long pain-free periods, whereas type B pain persists for a longer period of time (1-2 mo) with intervals of intense pain. Type A pain is experienced more often and is typically easier to treat. Several serum-based biomarkers have been identified for the diagnosis of acute pancreatitis such as amylase, lipase and trypsinogen^[181]. In acute pancreatitis, the level of amylase (glycoside hydrolase) is rapidly induced within 4 to 6 h of disease onset, remains high for 3 to 4 d and sensitivity decreases with time from onset^[182-184]. Higher levels of lipase have been found during the onset of acute pancreatitis, and it is more specific and sensitive than amylase for detecting acute pancreatitis because serum level of lipase remain elevated for around 2 wk before it returns to the normal level^[183,185]. The sensitivity and specificity of amylase is about 63.6% and 99.4%, whereas sensitivity and specificity of lipase were 95.5% and 99.2%, respectively^[186,187]. Pancreatic lipase is four times more active than amylase and it is less affected by exocrine pancreatic deficiency occurring in patients with chronic pancreatitis^[183,188]. Trypsinogen is the inactive form of the enzyme trypsin and is cleaved by duodenal enterokinase to produce the active enzyme trypsin and trypsinogen activated peptide^[183,189]. Normally trypsinogen is secreted in very low levels from pancreatic acinar cells but during pancreatitis secreted trypsinogen enzyme moves into the systemic circulation due to increased vascular permeability, and consequently there is increased clearance in the urine. During the onset of disease, trypsinogen concentration is elevated in the serum as well as urine and declines to normal level within 3 to 5 d^[183,185,190].

CURRENT TREATMENTS STRATEGY

The first-line of treatment involves fasting along with intravenous fluids if the pancreatitis is very painful and this help the pancreas to rest and recover. Depending on the underlying cause of pancreatitis, management may vary to address the specific cause. Currently, several medications and treatment options are available

such as analgesics like paracetamol or non-steroidal anti-inflammatory drugs or both followed by tramadol, perhaps coupled with a neuroleptic antidepressant. Another option is steroid therapy in which prednisolone is used for the treatment of autoimmune pancreatitis^[191,192]. Furthermore, micronutrient therapy seems to be promising and it includes vitamin C, E, B6, B12, folic acid, methionine, and β -carotene. Braganza *et al.*^[10] have revealed that micronutrient therapy is designed to supply methyl and thiol moieties, which are helpful to restrict the generation of reactive oxygen species and deactivate pro-inflammatory oxidation products, reduce mast cell degranulation, decrease necrosis of pancreatic acinar cells and lessen pro-fibrotic induction. The outcome of these six clinical trial-based studies revealed that micronutrient therapy controls the pain and curbs attacks in patients suffering with chronic pancreatitis^[193-199]. If pancreatitis is due to an obstructing gallstone, surgical intervention may be needed to remove the gallstone. Intervention may also be required to treat a pseudocyst or surgically remove the part of affected pancreas. Micronutrient treatment seems to substantially reduce the need for surgery.

CONCLUSION

The current review provides a comprehensive understanding of the development of chronic pancreatitis and the role of cells and cytokines involved in promoting pathogenesis. Briefly, we have discussed disease characteristics, molecular mechanisms involved in pancreatitis, the role of granulocytes such as neutrophils and eosinophils, the details of associated cytokines and chemokines implicated in the progression along with major signaling pathways such as TGF- β /SMAD, MAP kinase, PI3K, Rho kinase, and JAK/STAT that are crucial in the development of pancreatic fibrosis following pancreatic injury. This review will help to understand the intricate process of several autocrine and paracrine pathways involved in pancreatitis pathogenesis including remodeling. We provided details regarding the disease that might be useful for investigators to focus on, and cells and their associated mediators that might be helpful for future strategies for diagnostic and therapeutic interventions in the treatment of pancreatitis.

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Psychotropic drugs and liver disease: A critical review of pharmacokinetics and liver toxicity

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Abstract

The liver is the organ by which the majority of substances are metabolized, including psychotropic drugs. There are several pharmacokinetic changes in end-stage liver disease that can interfere with the metabolism of psychotropic drugs. This fact is particularly true in drugs with extensive first-pass metabolism, highly protein bound drugs and drugs depending on phase I hepatic metabolic reactions. Psychopharmacological agents are also associated with a risk of hepatotoxicity. The evidence is insufficient for definite conclusions regarding the prevalence and severity of psychiatric drug-induced liver injury. High-risk psychotropics are not advised when there is pre-existing liver disease, and after starting a psychotropic agent in a patient with hepatic impairment, frequent liver function/lesion monitoring is advised. The authors carefully review the pharmacokinetic disturbances induced by end-stage liver disease and the potential of psychopharmacological agents for liver toxicity.

Key words: Liver; Toxicity; Psychotropic drugs; Pharmacokinetics; Hepatic disease

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Core tip: The liver is the organ by which the majority of substances are metabolized, including psychotropic drugs. There are several pharmacokinetic changes in end-stage liver disease that can interfere with the metabolism of psychotropic drugs. The evidence is insufficient for definite conclusions regarding the

prevalence and severity of psychiatric drug-induced liver injury. High-risk psychotropics are not advised when there is pre-existing liver disease, and after starting a psychotropic agent in a patient with hepatic impairment, frequent liver function/lesion monitoring is advised.

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INTRODUCTION

Among all of the organs in the human body, the liver performs the greatest number of functions. The liver's multiple activities are important and have impacts on all body systems, including the nervous system. It is also in the liver that most of the substances that we ingest are metabolized, including drugs.

Liver failure occurs when large parts of the liver become damaged beyond repair, and the liver is no longer able to function. Drug-induced liver injury (DILI) is the 4th most important cause of liver disease in Western countries^[1]. The incidence of DILI is between 1/10000 and 1/100000 patients-years^[2,3].

The drugs used in psychiatry and neurology are the second most important group of drugs implicated in hepatotoxicity, after anti-infectious drugs^[4]. The hepatic reserve is reduced in patients with cirrhosis or chronic hepatic failure, and when DILI occurs in such patients, it can be more severe^[5]. Therefore, high-risk drugs should be contraindicated in cases of pre-existing liver disease^[6].

Conversely, liver failure interferes with different stages of drug pharmacokinetics: Absorption, metabolism, distribution and elimination. Therefore, it affects drug concentrations, duration of action, and effectiveness. It is essential to be aware of these processes and consequent changes in the circulating concentrations of psychiatric drugs to prevent drug toxicity.

Psychiatric symptoms in patients with end-stage liver disease can occur due to co-existing psychological or physiologic processes (e.g., liver failure, encephalopathy, adjustment reactions to the stress of severe medical illness, etc.). All of these situations must be treated, not only with psychological interventions but also with psychotropic drugs. In these cases, patients with end-stage liver disease require special concern because they are medically vulnerable and are at increased risk for medication-induced adverse reactions.

The purpose of this paper is to review the evidence regarding fundamental pharmacokinetic alterations caused by end stage liver disease as well as the potential for liver toxicity with psychopharmacological

agents. In our review, we analyse the evidence for DILI, severe liver injury leading to death or liver transplantation, abnormalities of liver function tests in clinical trials and hepatotoxicity. Finally, we provide several recommendations and directions regarding the psychotropic drugs that require special attention and how to minimize the risks of liver toxicity.

PHARMACOKINETIC CHANGES IN END-STAGE LIVER DISEASE

Liver failure can affect some aspects of medication pharmacokinetics, ranging from absorption to distribution and elimination. We discuss the most important pharmacokinetic processes that might lead to increased drug concentrations in liver disease patients.

Distribution

In end-stage liver disease, a great part of the blood in the portal vein escapes from the liver and flows straight into the systemic circulation (by means of portosystemic shunts). This process is due to intra- and extra-hepatic shunts that can occur in these patients. Therapeutic shunts (surgical and angiographic) can also be used to alleviate portal hypertension^[7].

These shunts can affect first-pass metabolism by diminishing liver perfusion. In these cases, less drug passes through the liver before systemic distribution. Consequently, there is an elevation in drug concentrations in the blood. This effect is particularly important for drugs with extensive first-pass metabolism (Table 1). The pharmacokinetics of other psychotropic drugs, such as diazepam and paroxetine, with less affinity for liver enzymes, are not as influenced by first-pass metabolism^[8].

Although olanzapine has great first-pass metabolism, it is mostly metabolized by second-phase liver metabolic processes (preserved in liver disease), so it might not be an important factor for this particular drug^[9].

Protein binding

More than 80% of psychiatric drugs are bound to plasma proteins, such as lipoproteins, alpha₁-acid-glycoprotein and albumin. Some psychotropic drugs, such as fluoxetine, aripiprazole and diazepam, are highly protein bound. Nevertheless, there are some psychotropic drugs that minimally bind to proteins, such as venlafaxine, lithium, topiramate, gabapentin^[10], pregabalin, methylphenidate and memantine^[11-17].

The cirrhotic liver produces a smaller quantity of albumin and alpha₁-acid-glycoprotein, which is conducive to an increased concentration of free active drug in the blood^[18,19].

This increase is particularly important for highly protein-bound drugs, such as benzodiazepines (particularly diazepam, which is more than 99% protein

Table 1 Psychotropic drugs with extensive first-pass metabolism^[10-16]

Tricyclic antidepressants - first-pass metabolism greater than 50% after oral administration
SNRI antidepressants - venlafaxine
SSRI antidepressants - sertraline
NRI antidepressants - bupropion
Typical antipsychotics - chlorpromazine
Atypical antipsychotics - olanzapine (40%), quetiapine

SSRI: Selective serotonin reuptake inhibitors; SNRI: Serotonin and norepinephrine reuptake inhibitors; NRI: Norepinephrine reuptake inhibitors.

bound^[20]. Therefore, in cirrhosis, the side effects that result from the administration of these drugs, such as sedation, can be more severe.

Metabolism

Some psychotropic drugs are water-soluble and are directly removed from the circulation in the urine and bile, which is the case with lithium, gabapentin, and topiramate^[10]. However, all of the other psychotropic drugs are lipid soluble and must be metabolized in the liver, where they undergo some chemical changes and become more soluble. Only then can they be excreted in the urine or bile.

The metabolic reactions that take place in the liver can occur in two main phases^[19]. In phase I, cytochrome P-450 enzymes (monooxygenases) are responsible for the hydrolysis, oxidation, dealkylation or reduction of the molecule. Most of the time, these reactions decrease the pharmacological activity of the substrate. However, drugs are sometimes metabolized into active metabolites, which is the case with some benzodiazepines (such as diazepam, chlordiazepoxide), tricyclic antidepressants (such as amitriptyline and imipramine) and antipsychotics (such as chlorpromazine, thioridazine, risperidone)^[10,21]. In phase II, liver enzymes are responsible for the conjugation of the drug with an endogenous molecule, such as glucuronic acid, sulphate, amino acids, acetate or glutathione. This process renders the original molecule more hydrophilic^[19], and in most of the cases, it eliminates all of the pharmacological activity.

Conjugation with glucuronic acid (glucuronidation) is normally preserved in liver disease^[21]. Therefore, it might be beneficial to select a psychiatric drug that only requires glucuronidation (and does not require a phase I reaction), which is the case with temazepam, oxazepam, and lorazepam^[8,9,19]. Olanzapine also requires almost only glucuronidation in its metabolism^[9].

Fluid status

Although it is believed that water-soluble drugs, such as lithium, are safe to use in liver disease patients, there are some aspects that must be considered.

In fact, it is not easy to maintain therapeutic serum levels of drugs such as lithium with the changes in fluid status that can occur in liver disease patients.

These changes can be due to possibly abnormal renal haemodynamics (which often occur in liver disease patients) but also to any sudden change in fluid status that can occur due to some therapeutic procedures (such as paracentesis, extreme diuresis, or diarrhoea induced in the treatment of liver encephalopathy).

If the total volume of body fluid is suddenly reduced, the regular therapeutic drug level can become critically toxic. Therefore, when using these types of drugs (such as lithium) in patients with cirrhosis, a strict coordination is mandatory between the different medical specialists that assist the patient^[10,17].

DILI

DILI can be classified depending on different criteria: Underlying injury; pathophysiological mechanism; clinical evolution; and severity of the lesion. Each of these criteria are reviewed.

Underlying liver injury

DILI can be classified into three main categories according to the pattern of liver injury (*i.e.*, hepatocellular and cholestatic or mixed). Hepatocellular injury accounts for 90% of drug-induced hepatotoxicity and is associated with abnormally high serum alanine aminotransferase (ALT) titres, with a small or no increase in alkaline phosphatase (ALP) titres; an associated high serum bilirubin level, found in cases of severe hepatocellular damage, is a marker for poor prognosis^[22]. Cholestatic liver injury is associated with high serum ALP titres only slightly higher than normal ALT levels; serum bilirubin concentrations might also be high. In cases of mixed injury, both ALT and ALP levels are abnormally high.

Another type of lesion is steatosis. This reaction is generally chronic and occurs with gradual and increased fat accumulation in the liver (especially triglycerides), which can be caused by different situations, including the use of certain drugs. In drug-induced steatosis (almost always reversible), benign macrovacuolar steatosis can become steatohepatitis and cirrhosis in some cases^[23].

Steatosis can occur with exposure to some antipsychotics (*e.g.*, clozapine, olanzapine) and antiepileptics (*e.g.*, valproate)^[23-28]. Less frequently, steatosis can be microvesicular, consistent with a more serious form of fat deposition in the hepatocytes, associated with more severe and acute clinical consequences (*i.e.*, valproate or Reye's syndrome).

Pathophysiological types of DILI

Two pathophysiological types of DILI have been identified.

The more common type is idiosyncratic, dose independent and unpredictable^[29]. It is the consequence either of immune-mediated liver damage (immunoallergic idiosyncratic DILI) or of direct cellular injury (metabolic idiosyncratic DILI)^[30]. A hypersensitivity syndrome (fever,

rash, eosinophilia, auto-antibodies) and a short latency period (1-6 wk)^[30] suggest immune-mediated hepatic injury, whereas the absence of any hypersensitivity syndrome and a longer latency period (1 mo to 1 year) suggest an idiosyncratic metabolic mechanism^[31]. Intrinsic DILI, related to drug accumulation, has also been described; it is dose dependent and predictable and has generally been observed during preclinical and clinical trials, leading to early drug withdrawal.

Clinical evolution (acute/chronic)

DILI can be acute or chronic, depending on clinical presentation. Acute DILI is the most common form of DILI, accounting for 10% of all cases of acute hepatitis. Histologically, it can present as acute hepatitis, cholestatic injury, a mixed pattern or acute steatosis. Chronic DILI is defined as persistence of abnormal liver enzymes for > 6 mo, and it accounts for 10% of DILI cases, more often following acute cholestasis. It can resemble other causes of chronic liver disease, such as autoimmune hepatitis or alcoholic liver disease^[32].

Severity of DILI

Regarding its severity, DILI can be mild, severe and fatal.

According to the Drug-Induced Liver Injury Network (DILIN), in mild DILI, there is elevation of ALT and/or alkaline phosphatase, but no important increases in bilirubin and no impairment of coagulation. In severe DILI, there is elevation of ALT and/or alkaline phosphatase, bilirubin is also increased, and one or more of the following exists: Extended jaundice for more than three months; and liver or other organ failure (induced by the drug). In fatal DILI, death occurs if the patient does not undergo liver transplantation^[33].

The available data show that all psychotropic agents are associated with a risk of hepatotoxicity^[34]. Most of the cases of DILI are mild, and liver tests normalize after drug withdrawal. Nevertheless, sometimes the consequences are very severe, leading to death or liver transplantation.

The most important means of assessing the potential for a psychotropic drug to cause severe or fatal hepatic injury is to review the published case reports. Nevertheless, there is no way to determine incidence rates, and the inexistence of case reports cannot be interpreted as the medication being free of risk regarding severe or fatal DILI. Conversely, the risks with different medications cannot be compared by this methodology because they are prescribed in different rates, and they have existed for different periods of time. For example, the probability of having case reports for older drugs is much higher than for newer ones^[35].

Another problem is that, in many cases of reported DILI for a certain drug, the patient has co-medications and several medical co-morbidities.

Detection of DILI during premarketing clinical trials is a difficult challenge because of the small numbers of

patients treated and the short duration of the majority of clinical trials (6-12 wk) relative to the latency of DILI^[36,37].

Antidepressants

Antidepressant-associated DILI is generally of the hepatocellular type and less frequently of the cholestatic or mixed type^[31-34]. Concerning pathophysiology, it can be immunoallergic or metabolic. Various biological and clinical presentations are possible, ranging from isolated increases in liver enzyme levels to loss of hepatocellular function, acute liver failure, and death^[38].

Based on severity and frequency of liver injuries reported for the different antidepressants, Voican classified the agents as high risk and lower risk. High-risk agents include tricyclic antidepressants (imipramine, amitriptyline) and nefazodone (which has been withdrawn from the market in several countries, due to 55 severe cases of DILI reported, including 20 deaths), as well as venlafaxine, duloxetine, sertraline, bupropion, trazodone, and agomelatine^[22,38-42].

Drugs with apparently lower risks are citalopram, escitalopram, paroxetine and fluvoxamine^[38,43].

Gahr *et al.*^[44] confirmed the results of Voican's comprehensive review using an innovative method. They calculated and compared reporting odds ratios, based on the number of adverse drug reactions related to hepatic disorders/total number of adverse drug reaction among several antidepressants^[44].

Regarding agomelatine (AGM), there is disagreement between the pervasive idea that this antidepressant might have a great risk of liver toxicity and the availability of published data providing this evidence perhaps because of the short life of this antidepressant^[44].

However, in a recent EMA (European Medicines Agency) post-authorization opinion, AGM was reported to be associated with a high hepatotoxic risk, and some limitations on its use were suggested. Clinical trials have shown a higher prevalence of increased ALT in patients treated with AGM (1.34% on AGM 25 mg/d, 2.51 on AGM 50 mg/d), compared to placebo (0.5%). Moreover, since the marketing authorization for AGM in 2009, several cases of severe liver injury-associated with AGM have been reported^[6,30].

These cases indicate that AGM should be avoided in patients with pre-existing liver function compromise. Furthermore, it is recommended by the company responsible for this drug that regular laboratory analysis be performed in cases of prescription of AGM. If there is treatment-associated elevation of liver enzymes, AGM should be rapidly discontinued. Patients of female sex, who are older than 50 years of age, and who are poly-medicated can have increased risk of liver toxicity related to AGM, although there is still only scarce regarding these matters. More studies are expected in this field, and they could likely affect the actual recommendations regarding AGM^[6,44]. Table 2 summarizes the data on

Table 2 Antidepressants and liver toxicity

	Epidemiology	Type of lesion	Mechanism
Tricyclic antidepressants			
Imipramine	ALT transient elevation-20% ^[45] Cholestatic jaundice: 0.5%-1% ^[2] DILI: 4/100000 patient-years ^[2,46] Fatal/Trxp DILI: 1 ^[47]	Hepatocellular, cholestatic	Immuno-allergic
Amitriptyline	ALT transient elevation-10% ^[45] Abnormal LFT: 3% ^[48] Fatal/Trxp DILI: 1 ^[39]	Hepatocellular, cholestatic	Immuno-allergic
Clomipramine	Severe DILI: 2 reports ^[42,49]	Hepatocellular	Immuno-allergic
MAO inhibitors			
Moclobemide	Abnormal LFT: 3% ^[50] Fatal DILI: 1 ^[51]	Hepatocellular, cholestatic	Immuno-allergic
Serotonin-norepinephrine reuptake inhibitors			
Venlafaxine	ALT > 3ULN: 0.4% ^[52] Severe DILI: 6 ^[53-56] Fatal DILI/Trxp: 1 ^[57]	Hepatocellular, cholestatic	Immuno-allergic, metabolic
Duloxetine	ALT > 3ULN: 1.1% ^[58] ALT > 5ULN: 0.6% ^[59] DILI: 26.2/100000 patient-years ^[60,61] Severe DILI-7 ^[5] Fatal/Trxp DILI: 13 ^[60]	Hepatocellular, cholestatic, mixed	Immuno-allergic, metabolic
Serotonin-reuptake inhibitors			
Sertraline	ALT > 3ULN: 0.5%-1.3% ^[46] DILI: 1.28/100000 patient-years ^[46] Severe DILI: 4 ^[62-65] Fatal/Trxp DILI: 2 ^[66,67]	Hepatocellular, cholestatic, mixed	Immuno-allergic, metabolic
Paroxetine	ALT > 3ULN: 1% ^[46] Severe DILI: 4 ^[68-71]	Hepatocellular, cholestatic, chronic hepatitis	Metabolic
Fluoxetine	ALT > 3ULN: 0.5% ^[46] Severe DILI: 6 ^[72-77]	Hepatocellular, cholestatic, chronic hepatitis	Metabolic
Fluvoxamine	Unknown ^[38] DILI: 3 ^[78-80]	Hepatocellular	Metabolic
Citalopram, escitalopram	No difference in LFT <i>vs</i> placebo ^[81,82]	?	?
Other antidepressants			
Nefazodone	DILI: 28.96/10000 patient-years ^[38] Severe DILI-35 ^[83] Fatal-20 ^[83]	Hepatocellular, cholestatic, mixed	Metabolic
Trazodone	ALT > 3 unknown ^[38] Severe DILI-7 ^[84] Fatal/Trxp DILI-2 ^[57,85]	Hepatocellular, cholestatic	Immuno-allergic
Bupropion	ALT > 3ULN: 0.1%-1% ^[86] Severe DILI: 3 ^[86-88] Fatal/Trxp DILI: 2 ^[89,90]	?	?
Agomelatine	ALT > 3ULN: 1.4% (25 mg/d) ALT > 3ULN: 2.5% (50 mg/d) ^[6,91] Severe DILI: 6 reports ^[92,93] Fatal/Trxp DILI: 1 ^[94]	Hepatocellular	
Mirtazapine	ALT > 3ULN: 2% ^[95] Severe DILI 2: reports ^[96]		

DILI: Drug-induced liver injury; ALT: Alanine aminotransferase; LFT: Liver function tests; ULN: Upper normal limit.

hepatotoxicity of the main antidepressant drugs.

Antipsychotics

Cytochrome P450 (in the liver) is responsible for the metabolism of most antipsychotics (excluding sulpiride, amisulpride, and paliperidone)^[97,98]. Antipsychotics can induce liver injury by means of three main mechanisms: Hepatocellular, cholestatic and steatosis.

Typical antipsychotics: The risk of hepatotoxicity with chlorpromazine is well established^[34].

The main mechanism by which chlorpromazine and other phenothiazines induce cholestatic disease remains unclear. The existence of eosinophilia and rash during its early onset (frequently 1 mo) and that there is not a dose relationship for its toxicity reveal that the mechanism could be some type of hypersensitivity. Nevertheless, some authors have indicated that its toxicity might be related to an idiosyncratic metabolic reaction that depends on individual sensitivity^[2]. The bile duct can be the most affected, and as a consequence, a severe ductopenic syndrome can occur^[2].

Table 3 Antipsychotics and liver toxicity

	Epidemiology	Type of lesion	Mechanism
Typical			
Chlorpromazine	Jaundice: 0.16%-0.3% ^[99] Severe DILI: > 350 ^[100,101,115-124] Fatal Injury: 8 ^[102-109]	Cholestatic	Immuno-allergic
Haloperidol	ALT > 3ULN: 2% ^[110] Severe DILI: 1 ^[111]	Cholestatic	Immuno-allergic
Atypical			
Clozapine	ALT > 3ULN: 15% ^[125] Severe DILI: 16 ^[126-140] Fatal Injury: 2 ^[141,142]	Hepatocellular Cholestatic Chronic esteatosis	Immuno-allergic Chronic estatosis
Olanzapine	ALT > 3ULN: 6% ^[143] Severe DILI: 7 ^[139,144-149]	Hepatocellular Cholestatic Chronic esteatosis	Immuno-allergic, Chronic estatosis
Risperidone	ALT > 3ULN: 3% ^[150] Severe DILI: 13 ^[150-162]	Hepatocellular Cholestatic Chronic esteatosis	Immuno-allergic Chronic estatosis
Quetiapine	ALT > 3ULN: 0% ^[143] Severe DILI: 3 ^[151,163,164] Fatal injury: 2 ^[165,166]	?	?
Ziprasidone	Not reported Severe DILI: 1 ^[167]	?	?
Aripiprazole	Not reported		
Amisulpride	Not reported		

DILI: Drug-induced liver injury; ALT: Alanine aminotransferase; ULN: Upper normal limit.

A study that reviewed prescriptions in the United Kingdom between 1985 and 1991 showed a total incidence of chlorpromazine jaundice of 0.16% (more elevated in patients who were older than 70 years old, 0.3%)^[99].

Severe DILI was reported in more than 350 cases^[100,101], and fatal injury in 8 cases^[102-109].

Haloperidol, while structurally similar to the phenothiazines, rarely causes severe liver compromise. When it occurs, the mechanisms of liver toxicity are similar to those of phenothiazines (cholestatic lesions)^[2]. A frequency of elevated liver enzymes of 2%^[110] was reported, but only 1 case of severe DILI was reported^[111].

Atypical antipsychotics: Atypical antipsychotics rarely induce severe liver toxicity. Nevertheless, asymptomatic increases in the levels of liver enzymes and bilirubin are not uncommon when using these psychotropic drugs. In most cases, the laboratory changes appear after 6 wk of treatment, and they tend to disappear and not worsen^[35].

The type of hepatic lesion associated with antipsychotics can follow a primary hepatocellular pattern; therefore, the main change in laboratory tests seems to be an elevation in aminotransferases^[35]. Nonalcoholic fatty liver disease can also be associated with treatment with atypical antipsychotics *via* metabolic syndrome, which they can induce^[112].

Hence, many authors have advocated that it is important to assess liver function tests before initiating treatment with atypical antipsychotics, and subsequently, routine control of aminotransferases must

be performed. Checking every year (and 6/6 mo in the case of clozapine) has been recommended^[113]. In patients with heavy use of alcohol or other substances, more frequent control might be necessary. In this latter group of patients, it is also recommended to be more careful with slight changes in laboratory tests. If signs of liver compromise (*e.g.*, jaundice, pruritus, nausea, anorexia, *etc.*) are present, laboratory tests should be assessed at once.

The antipsychotic should be stopped if there is an asymptomatic increase in aminotransferases higher than 3 times the maximum level of normal (aminotransferases are sensitive marker of liver injury)^[114].

It is necessary to pay special attention to patients with pre-existing hepatic disease or patients treated with other drugs that can be aggressive to the liver. Because the majority of atypical antipsychotics are relatively new, there still are no long-term hepatic follow-ups with some of these drugs. Therefore, new evidence might appear in longer controlled studies regarding the frequency of and risk factors for liver damage^[108].

In his comprehensive review, Marwick stated that LFT abnormalities in adults receiving regular antipsychotics are "common, early, mild, and often transient"^[35]. Severe or fatal DILI is very rare. Chlorpromazine is the antipsychotic most associated with severe liver toxicity and therefore should not be used in patients with pre-existing liver dysfunction^[35]. Among the atypical antipsychotics clozapine, is the antipsychotic most associated with LFT abnormalities, and aripiprazole, ziprasidone and amisulpride might be associated with

Table 4 Mood stabilizers and benzodiazepines and liver toxicity

	Epidemiology	Type of lesion	Mechanism
Antiepileptics			
Carbamazepine	Transient ALT, AST, GGT elevations: 61% patients 1%-22% ^[3] DILI: 1% ^[170]	Hepatocellular, cholestatic	++Hypersensitivity -- Metabolic
Valproate	Transient ALT, AST elevations: 10%-15% patients ^[170] Hyperrubirubinemia-44% ^[170] DILI: 3%-44% ^[173]	Hepatocellular	Metabolic (Toxic metabolites through w-oxidation)
Lamotrigine	Fatal DILI: 0.02% (0.2% children < 2a) ^[1] Transient ALT, AST elevations < 1% Rare hepatotoxicity ^[170] (4 severe DILI) ^[174]	Hepatocellular	Statisis Metabolic
Topiramate	Transient ALT, AST elevations < 1% ^[1] Rare hepatotoxicity (2 severe DILI) ^[174]	Hepatocellular	Metabolic
Gabapentine; pregabalin	Rare hepatotoxicity ^[1]	?	?
Benzodiazepines			
Chlordiazepoxide, diazepam, flurazepam	Rare hepatotoxicity ^[171,172]	Cholestatic	Hypersensitivity
Lithium	Very rare hepatotoxicity ^[1]	?	?

DILI: Drug-induced liver injury; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

Table 5 Pharmacokinetic changes caused by end-stage liver disease: Psychotropic drugs that require special attention

Avoid drugs with extensive first-pass metabolism	Avoid Tricyclic Antidepressants (first-pass metabolism 50%), venlafaxine, sertraline, bupropion, chlorpromazine, quetiapine
Avoid highly protein bound drugs	Avoid most psychotropic drugs (specially fluoxetine, aripiprazole and benzodiazepines). Except: Venlafaxine, lithium, topiramate, a gabapentin, a pregabalin, memantine
Avoid drugs depending on phase I hepatic metabolic reactions	Preferable: Lithium, gabapentin, topiramate, amisulpride (depending mainly on renal excretion) and some benzodiazepines (oxazepam, temazepam, lorazepam) that depend on phase II reaction or glucuronidation, which is preserved in cirrhosis

fewer LFT abnormalities. Table 3 summarizes the data about the hepatotoxicity of the main antipsychotics.

Mood stabilizers and benzodiazepines

The overall incidence of the hepatotoxicity of antiepileptics has been estimated at 1/26000 to 1/36000. The most used antiepileptic drugs in psychiatry are valproate, carbamazepine, topiramate, lamotrigine and gabapentin. Of these drugs, Valproate is associated with the greatest risk of potential liver toxicity. Gabapentin and pregabalin are the safest^[129].

Valproate hepatotoxicity is generally idiosyncratic. The period of treatment before the onset of the injury can range from 3 d to 2 years. The absence of hypersensitivity symptoms, the morphology of the DILI and the slow onset suggest that the idiosyncrasy is metabolic. It is more common in infants and children^[129].

Transient elevations of aminotransferases can be present in 10%-15% of patients and hyperbilirubinemia in up to 44%. Therapy can be continued as long the elevations in aminotransferases are less than 3 times the ULN. Sometimes, normalization of liver tests occurs likely because of adaptation^[168]. Regarding carbamazepine, hepatic adverse events are frequent but are most represented by transient asymptomatic elevations in liver tests (ALT, AST, GGT).

Severe liver damage caused by carbamazepine is infrequent, but it has a very typical presentation. One to eight weeks after beginning treatment with this drug, a hypersensitivity syndrome occurs, with fever, rash, facial oedema, lymph node enlargement, and leucocytosis (with eosinophilia)^[1,169].

Less frequently, carbamazepine-induced DILI can occur without immuno-allergic characteristics. In these cases, the resulting clinical syndrome has a late onset (up to 6 mo after initiating treatment)^[1,169].

Hypersensitivity is noted in up to 10% of patients. Hepatic adverse events have been reported to constitute 10% of all hypersensitivity reactions, for a total incidence of DILI due to carbamazepine hypersensitivity reactions of 1%^[170].

Elevations in occur in less than 1% of patients on lamotrigine. Hepatotoxicity is rare and idiosyncratic, and it typically exhibits a hepatocellular pattern of injury^[170]. The same outcome occurs with topiramate^[1].

Benzodiazepine-induced liver damage is rare, with few cases reported in the literature, generally with a cholestatic pattern^[171,172].

Long-term treatment with lithium can, in some cases, induce some LFT abnormalities. These changes are generally temporary and asymptomatic, reverting even if treatment continues. In cases of lithium over-

dose, these LFT changes can be marked, although the damage is much more severe in other organs, such as the kidney^[1]. Table 4 summarizes the data about the hepatotoxicity of the main mood stabilizers and benzodiazepines.

CONCLUSION AND GENERAL RECOMMENDATIONS

The available data on psychotropic drug-induced hepatic toxicity are mostly from reported cases and, to a lesser extent, from the results of clinical trials and other studies, especially for the most recent drugs. It is therefore difficult to draw conclusions about the prevalence and severity of DILI.

Regarding pharmacokinetic changes in end-stage liver disease, there are some psychotropic drugs that require special attention, as shown in Table 5.

It is likely that all psychopharmacological agents are associated with a risk of hepatotoxicity. However, the evidence is insufficient for rigorous conclusions to be drawn about the prevalence and severity of psychiatric DILI^[175].

Hepatic reserve is reduced in patients with cirrhosis or chronic hepatic failure, and when DILI occurs in such patients, it can be more severe^[5,176]. Therefore, high-risk drugs should be contraindicated in cases of pre-existing liver disease^[6] (based on comprehensive reviews).

Before starting a psychotropic agent, baseline laboratory testing (e.g., LFT, ALT) is recommended^[113,177]. If liver disease is present, it is preferable to use psychotropic drugs with minimal liver metabolism (e.g., topiramate, sulpiride and amisulpride)^[35]. High-risk psychotropic agents (referred to in comprehensive reviews, see above) are not advised when there is pre-existing liver disease. After starting a psychotropic agent in a patient with hepatic impairment, frequent liver function/lesion monitoring is advised^[113].

If a patient has normal laboratory tests (e.g., LFT, ALT) before initiating treatment, there is no clear unanimity regarding the frequency of analysis re-assessment. Laboratory tests with ALT > 3ULN or ALP > 2ULN are considered sensitive markers for liver damage, and in these cases, the psychotropic agent should be stopped^[35,114].

After starting a psychotropic agent, patients should be counselled to report signs and symptoms of liver dysfunction that could be associated with the use of their drug, including weight loss/decreased appetite, gastrointestinal problems or changes, dark (i.e., tea-coloured) urine, yellowing of eyes (i.e., jaundice), weakness, or unexplained/increasing fatigue. Other signs and symptoms include pruritus, clay-coloured stools, muscle pain, and increased confusion. Some of these conditions are already associated with chronic hepatitis infection, so it is important to emphasize observations of new-onset signs and symptoms. Patients and/or their caretakers should be encouraged

to report these observations to their clinicians should they occur at any time after starting a psychotropic agent. Prompt discontinuation of the suspected agent at symptom onset might decrease the likelihood of worsening progression, which can lead to permanent liver damage^[83].

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Update on clinical and research application of fecal biomarkers for gastrointestinal diseases

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Abstract

Gastrointestinal (GI) diseases comprise a large spectrum

of clinical conditions ranging from indigestion to inflammatory bowel diseases (IBDs) and carcinomas. Endoscopy is the usual method employed to diagnose these condition. Another noninvasive way to assess and diagnose GI conditions are fecal biomarkers. Fecal biomarkers provide information regarding a specific disease process and are perhaps more acceptable to clinicians and patients alike because of their non-invasivity compared to endoscopy. Aim of this review was to evaluate the current status of the fecal biomarkers in clinical and research for in GI diseases. Multiple types of fecal biomarkers are discussed in this review including; markers to assess IBD, which are released as a results of an inflammatory insults to intestinal epithelia such as antimicrobial peptides (lactoferrin) or inflammation related proteins (calprotectin). While markers related to function of digestion are primarily related to partially digested food or mucosal proteins such as abnormal amount of fecal fat α 1-antitrypsin, elastase and secretory IgA. The upcoming fecal biomarker like M2 pyruvate kinase and neutrophil gelatinase associated lipocalin are discussed as well. Apart from above mention, the fecal biomarkers under exploration for possible clinical use in future are also discussed. These include cathelicidins, osteoprotegerin, β -glucuronidase, Eosinophil proteins, *etc.*

Key words: Biomarkers; Gastrointestinal diseases; Inflammation; Lactoferrin; Calprotectin

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Core tip: There is a general inclination of clinicians as well as pathologists' to consider fecal biomarkers due to its non-invasivity. There are multiple types of fecal biomarkers in clinical use and under exploration for potential clinical use in future. It includes biomarkers for evaluating inflammatory bowel disease (*e.g.*, calprotectin, lactoferrin), for evaluating colorectal cancer, malabsorption and eosinophilic protein for allergic gastrointestinal diseases. In this review we have analyzed the current status in terms of their practical utilization of fecal

biomarkers with established indications and those which are under various stages of investigation.

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INTRODUCTION

A biomarker is an endogenous or exogenous substance measured in blood, plasma or urine whose levels correlate with disease occurrence or severity. Biomarkers are used to distinct a pathological condition from a physiological state and also monitoring treatment and disease progression. There is a general inclination of clinicians as well as pathologists' to consider fecal biomarkers due to its non-invasive nature with likely acceptability to the patient. Fecal biomarkers can be subdivided into following types based on their clinical application.

Markers of inflammatory bowel disease

These include inflammation related proteins, released during an inflammatory process in the gastrointestinal (GI) tract (*e.g.*, calprotectin) or antimicrobial peptides (*e.g.*, lactoferrin).

Biomarker of colorectal cancer

These are found in undifferentiated tissues and cells with increased expression in rapid turnover of such cells, *e.g.*, M2-pyruvate kinase.

Biomarkers for evaluation of malabsorption

Multiple markers are identified; most are undigested food particles like fecal fat globules, enzymes like α 1-antitrypsin and elastase for malabsorption assessment.

Biomarker for GI allergic diseases

Eosinophil related proteins are either released by or related to eosinophils and have application in assessing allergic and parasitic infestation of GI tract.

Biomarkers of gut health

This is an interesting group of biomarkers which with point toward the overall health of the gut mucosa. These biomarkers assess the integrity of gut barrier proteins and microbial fermentation products which are produced while fermentation of dietary particles by bacteria produces various chemicals, few of which such as short chain fatty acids, are used as biomarkers.

Different types of fecal biomarkers for GI diseases in clinical use and under investigations are shown in Tables 1 and 2. Although evidence for the newer markers is growing, currently only few fecal biomarkers have

achieved a place in routine clinical practice notably calprotectin is on top of that list. Out of the multiple fecal biomarkers with emerging roles in clinical use for GI diseases, only some are extensively studied for their clinical and diagnostic utilities. In this review we aim to provide an overview of current status of fecal biomarkers for GI diseases with established value in clinical and potential for future use.

METHODOLOGY

A search of databases PubMed, MEDLINE and Google Scholars was performed using the search terms "fecal biomarkers" and "gastrointestinal disease biomarkers". We selected articles written in English, published since 1990 in peer-reviewed journals, excluding reviews. The articles were then reviewed by two pathologists and one gastroenterologist keeping in view the ideology behind this review and relevant articles selected. This review is divided in two parts, in part one we have aimed to include diagnostic accuracies for the established markers in clinical use and in second part the clinical applications of fecal biomarkers under investigation for GI diseases are discussed.

Markers of inflammatory bowel disease

Calprotectin (S100A8/S100A9) and S100 A12 proteins: The S100 proteins are a family of calcium-binding proteins specifically linked to innate immune functions by their expression in phagocytes, monocytes, macrophages and granulocytes. The calprotectin is a heterodimer of S100A8 and S100A9. These proteins are released by cells of innate immunity and GI epithelial cells in condition of inflammation. They limit the growth of bacteria and fungi by sequestering manganese and zinc.

Calprotectin is a marker to diagnose or monitor inflammatory bowel disease (IBD), presently considered a gold standard and also included in clinical practice guidelines. It is reported to perform better than S100-A12 in diagnosing IBD and its levels correlate with the severity of IBD. Calprotectin is observed to perform better in predicting ulcerative colitis than Chron's disease^[1,2]. Meta-analysis have reported that calprotectin perform better in adults (sensitivity 93% and specificity 96%) than children (sensitivity 92% and specificity 76%)^[3]. In contrast there are conflicting studies regarding diagnostic utility of the S100A12 as an inflammatory marker and it have moderate performance compared to other inflammatory markers^[4-6].

Calprotectin is resistant to bacterial degradation in the gut and is stable in stool for up to one week at room temperature and is readily measured using immunochemical techniques. Limitations and diagnostic accuracies are conversed in Table 1.

Lactoferrin: Lactoferrin, an iron binding glycoprotein secreted in body fluids and produced by neutrophils,

Table 1 Fecal biomarkers for gastrointestinal diseases in clinical use with established diagnostic accuracies

S#	Name	Indication	Limitations	Sensitivity	Specificity
Biomarkers of IBD					
1	Calprotectin	Distinguishing functional from organic bowel disease and predicting relapse in IBD	Disease nonspecific Affected by age, comorbidities, NSAIDs use	70%-100%	70%-100%
2	S100 proteins	Inflammatory marker for IBD	Day to day variations	60%-67%	70%-90%
3	Lactoferrin	Markers of inflammation, Distinguish between IBS and IBD	Miss low level inflammatory activity Nonspecific marker of inflammation Raised in breastfeeding infants Cannot predict low level inflammation	67%-87%	90%-100%
Biomarker of cell turnover					
4	M2-PK	Screening of gastrointestinal tract cancers	Also raised in inflammation	67%-93%	88%-92%
Biomarkers of digestion and malabsorption					
5	Elastase-1 (e1)	Pancreatic insufficiency	Low specificity, also affected by other intestinal disorders	100%	96%
6	Fecal fat	Liver damage, hypolipidemic drugs, impaired gallbladder function, Celiac disease, Small bowel bacterial overgrowth	Cannot predict severity of disease Cannot be performed in diarrhea Not accurate or specific test	70%-94%	80%-99%
7	A1-antitrypsin	Protein-Losing Enteropathy, Whipple lipodystrophy, gastric carcinoma, intestinal lymphangiectasia	Nonspecific marker. Levels affected by inflammation	60%-78%	80%-85%

IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; NSAID: Nonsteroidal anti-inflammatory drug.

mononuclear phagocytes and epithelial cells, Figure 1. It limits the growth of bacteria by limited availability of iron and causes direct damage to bacterial cell membrane leading to its bactericidal activity. This glycoprotein is stable in feces as it is resistant to proteolysis and can be measured by immunochemical methods. These glycoproteins are released in excess amounts by neutrophils and phagocytic cells after inflammation, making it a unique marker of inflammation^[7]. Commercial assay for fecal lactoferrin measurement are now available based on immunochemical methods.

This is considered a good marker for evaluating IBD subjects, while evaluation as marker to distinguish between IBD and irritable bowel syndrome (IBS) there remains question marks, due to differences in results reported by different studies^[8-11].

Cathelicidins: These are small cationic antimicrobial peptides like defensins and are produced by neutrophils and epithelial cells of GI tract, released upon stimulation of these cells during infection. These peptides exhibit antimicrobial activity against GI pathogens, gram-negative and positive bacteria by disrupting microbial membrane integrity, Table 2. These peptides play a vital role in maintaining the balance between the GI luminal bacteria and antibacterial peptides, which is crucial for a healthy GI tract. Studies have reported this balance is disturbed in various disease states. However the role of these peptides as the cause or consequence of disease state is still unknown. They could participate in the development of different disorders ranging from inflammation to cancer.

Schauber *et al.*^[12] reported that colonic expression of cathelicidin is increased in ulcerative colitis but not

Crohn's disease. A study looking at cathelicidin role in *Escherichia coli* O157:H7 infection in mice and subsequent renal damage found that its deficiency was associated with severe infection and renal damage^[13]. Another study by Sarker *et al.*^[14] reported that antimicrobial peptides cathelicidins expressions were decreased in rectal and colonic epithelia in shigellosis infection in rabbits along with decreased expression in epithelia of lung and trachea, a sign of systemic infection. They also observed the treatment with phenylbutyrate counteracted the decreased expression of cathelicidins in such patients offering a potential antimicrobial activity against shigella infection^[14].

Osteoprotegerin: Osteoprotegerin is member of the tumor necrosis factor receptor superfamily. It binds to the receptor activator of nuclear factor kappa B ligand (RANKL), which in turn has pro-inflammatory properties, Table 2. A study by Nahidi *et al.*^[15] examining the role of osteoprotegerin in pathogenesis of IBD reported that it induced gut barrier deformities; increased permeability and decreased integrity of cell membrane along with loss of tight junctions; indicating that osteoprotegerin has pro-inflammatory effect and may contribute in pathogenesis of IBD^[15]. However the complete understanding of its function in IBDs needs further evaluation.

Beta-glucuronidase: Beta-glucuronidases enzymes secreted by lysosomes of colonocytes and certain bacteria, *e.g.*, *E. coli*, belong to glycosidase family of enzymes. This enzyme catalyzes the complex dietary carbohydrates, like glycosaminoglycans heparan sulfate. They also deconjugate variety of drugs, toxins,

Table 2 Fecal biomarkers under investigation for evaluating gastrointestinal diseases

S#	Name	Source	Function	Indication	Limitations
Biomarkers of inflammatory bowel disease					
1	Cathelicidins	Secreted by Neutrophils, keratinocytes and epithelial cells of gastrointestinal tract, respiratory tract, urogenital tract	Antibacterial activity, modulate inflammation by altering cytokine response, chemoattraction of inflammatory cells in diseased tissues	Marker of inflammation (IBD) and Shigellosis	Antimicrobial peptides so also increased in GI infections
2	Osteoprotegerin	Member of the TNF receptor superfamily	Binds to RANKL and blocks its interaction with RANK	Marker of inflammation (IBD)	Plays a role in bone metabolism so levels are increased in bone diseases
3	Beta-glucuronidase	Produced by colonocytes Also produced by anaerobic gut bacteria (particularly <i>E. coli</i>)	Enzyme that breaks down complex carbohydrates Deconjugate glucuronide molecules from a variety of toxins, carcinogens, hormones, and drugs	Marker of inflammation (IBD)	False results in cases of GI bacterial infection
4	Neutrophil Gelatinase Associated lipocalin	Member of the lipocalin family, secreted by neutrophils	Immunomodulation. Attaches to and neutralizes bacterial formylpeptides	Marker of inflammation (IBD)	Also increased in GI infections like enterocolitis
Eosinophil related proteins					
5	Eosinophil Protein X	When lamina propria is damaged, eosinophils migrate into the gut lumen Released by eosinophil; contribute to ongoing inflammation and tissue destruction	Marker of Eosinophil activity, Allergic and Parasitic influences	IgE-mediated food allergy Intestinal parasitic infection IBD	Also increased in GI inflammation
Biomarker of cell turnover					
6	Defensins	Expressed by neutrophils, epithelial and mucosal lining cell in small and large intestine	Antimicrobial peptide	Markers of colorectal cancer	Also raised in inflammation
Biomarkers of gut health					
7	Fecal secretory IgA	Secreted from mucosal surfaces	Gut epithelial barrier; Defense against the entry of enteric toxins and pathogenic organisms Development of immune tolerance of normal commensal gut organisms	Evaluate immunological response to intestinal pathogens Colorectal cancer	Cannot be used in subjects with immunoglobulin deficiency
8	SCFAs	Products of fermentation by colonic microbial flora; common ones are propionate, acetate, and butyrate	Provides 60%-70% of colonocytes energy requirements Lower colonic pH	Marker of inflammation (IBD)	< 5% of SCFA produced is excreted in stool Also levels altered by diet and rate of transit

SCFAs: Short-chain fatty acids; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease; GI: Gastrointestinal; RANK: Receptor activator of nuclear factor kappa B ligand.

hormones and also bilirubin in gut and are considered culprit of breast milk related jaundice in neonates, Table 2.

A study by Mroczynska *et al.*^[16], done on IBD and healthy children, reported that beta-glucuronidase activity was decreased by two times in children with IBD compared to healthy group. While in another study Manoj *et al.*^[17] reported that reduction in activity of intestinal as well as decreased levels of fecal beta-glucuronidase by using dietary fibers isolated from coconut or black gram may potentially play a role in preventing the formation of colon tumors induced by the carcinogen 1,2-dimethylhydrazine. These debatable findings warrant that further research is needed to completely understand chemical basis of beta-glucuronidase function.

Neutrophil gelatinase associated lipocalin: These proteins are released by neutrophils in response to some bacterial peptides (formylpeptides), which initiate bacterial protein synthesis. The neutrophil gelatinase

associated lipocalin (NGAL) released into the gut lumen then binds with bacterial peptide and neutralizes it, stopping bacterial protein synthesis^[18].

NGAL is another important inflammation related fecal marker under investigation for potential clinical utility. Serum and urinary NGAL are considered established markers for acute kidney injury and few studies have shown its levels are elevated in IBD but the levels don't correlate with disease severity^[19]. Recently multiple studies have shown that fecal NGAL levels are raised in subjects with IBD and its levels are significantly associated with disease activity and severity^[20-22].

Fecal biomarker of colorectal cancer

Pyruvate kinase: M2-pyruvate kinase is a dimer of pyruvate kinase; an enzyme involved in glycolysis pathway and plays an important role in tumor metabolism. It has increased expression in undifferentiated tissues and cells with in rapid turnover cells.

Its main role is in predicting GI cancers, both bleeding and non-bleeding types. Studies have reported

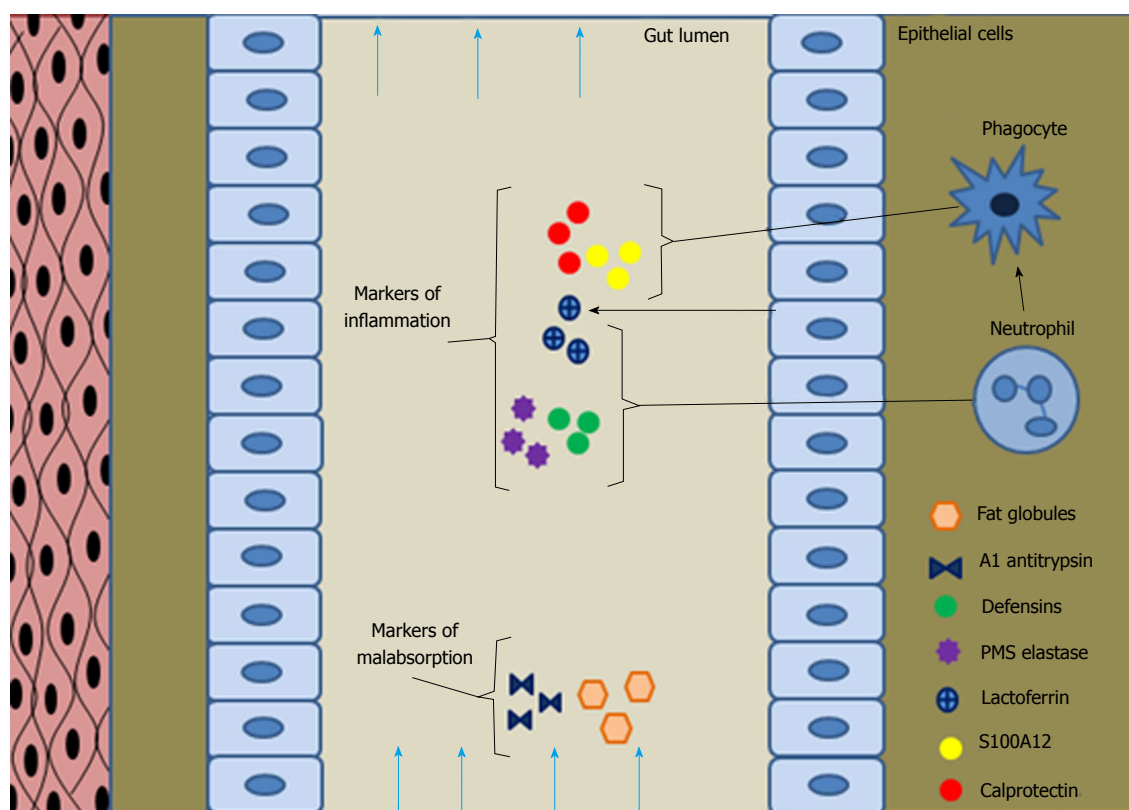


Figure 1 Overview of the potential source and clinical utility of various fecal biomarkers in clinical use.

it to be a marker in predicting colorectal cancers with good diagnostic accuracy, sensitivity and specificity of 93% and 97% respectively, Table 1^[23]. Currently it is being used for monitoring colorectal cancer subjects after treatment. Along with it, levels of M2-Pk are also elevated in breast, lung, ovarian, and thyroid cancers^[24]. One of its important limitations is that its levels are also increased in inflammation, so it should be used with caution in inflammatory conditions^[25].

Defensins: Defensins are small cationic antimicrobial peptides, classified into alpha and beta defensins on basis of their disulfide bond and sizes. These are expressed by neutrophils, epithelial and mucosal lining cell in small and large intestine, Figure 1. They play an important role in innate immunity; antimicrobial activity against bacteria, fungi and some enveloped viruses and the expression is induced by the pro-inflammatory cytokines and also through microorganisms.

As the name implies they were considered as markers of infectious and inflammatory GI diseases. However there is now accumulating evidence suggesting defensins as an evolving marker for evaluating colorectal cancers but there are controversial findings^[26-28]. Studies have also reported it to be an important marker for colorectal cancer. A study by Layton *et al*^[29] presented at American Association of Cancer Research 104th Annual Meeting in 2013 reported that β -defensins 1 was expressed in colon tissue samples of normal subjects while this expression was lost in subjects with

colorectal cancer. While another study by Melle *et al*^[30], reported that α -defensins are expressed more in colonic epithelium of patients with colorectal cancer than in normal epithelium, establishing defensins potential role as a tumor markers^[30]. So there remains a question mark regarding utility of this biomarker for evaluating colorectal cancer.

Biomarkers for evaluation of malabsorption

Elastase-1(e1) and PMN elastase: Serum elastase is a protease present in pancreatic secretion reaches the colon without being metabolized and is not affected by intestinal transit times or pancreatic enzyme replacement therapy, Figure 1. Elastase hydrolyzes denatured hemoglobin, casein, fibrin and albumin. It is a known biomarker for assessing exocrine pancreatic insufficiency, been in use for more than 3 decades. While its deficiency is associated with development of pulmonary emphysema and excess release results in hemorrhage due to vascular injury of acute pancreatic necrosis. Serum Elastase e1 levels are used for the diagnosis of acute or chronic pancreatitis, pancreatic insufficiency with good diagnostic accuracy^[31], Table 1.

Another type of elastase enzymes, the polymorphonuclear elastase (PMN-elastase) is secreted by neutrophils in response to inflammation^[32]. A study assessed the performance of calprotectin, lactoferrin and PMN-elastase in assessing IBD severity and differentiating between IBS and IBD, found that all these markers were able to differentiate active IBD

from inactive IBD as well as from IBS with diagnostic accuracies for lactoferrin, calprotectin and PMN-elastase of 80%, 80% and 74%^[33].

Fecal fat: Excess fat in the stool (steatorrhea) is often the first sign of fat malabsorption. This can be due to a number of factors, including chronic pancreatitis with or without stone obstruction, cystic fibrosis, neoplasia, Whipple disease, regional enteritis, tuberculous enteritis, celiac disease, or the atrophy of malnutrition, Table 1.

The fecal fat assessment is done by microscopy after sudan stain and is largely considered non-specific as it is affected by diet, discrepancies in sample collection, qualitative reporting and assay variation leading to lower diagnostic accuracy. To overcome these hurdles a new quantitative fecal fat microscopic method was introduced by Fine *et al.*^[34] in 2000, reported to have improved diagnostic accuracy; sensitivity of 94% and a specificity of 95% compared to the traditional method sensitivity and specificity of 76% and 99%, respectively. In this method they microscopically counted the fat globules of different diameter ranges (0-5 μm , 6-10 μm , 11-20 μm , 21-40 μm , 41-80 μm , and > 80 μm) in five high-power fields and the average number of each size range fat globules present were multiplied by the size-range midpoint. All products were then added to get a single fecal fat droplet total size number product. They reported that results obtained by this method correlates well with chemically measured fecal fat output and has a high diagnostic accuracy.

Alpha-1-antitrypsin: Alpha-1-antitrypsin a protease inhibitor is produced by the liver, macrophages, and intestinal epithelium and is resistant to degradation by digestive enzymes. Therefore offers utility for use as a biomarker in assessing the proteins loss distal to the pylorus. Protein loss is associated in certain GI conditions such as gastroenteritis and sprue. Alpha-1-antitrypsin can readily be measured by using commercially available assays, Table 1.

Fecal alpha-1-antitrypsin clearance has been a marker of clinical disease severity in IBDs for many years^[35]. Although α 1-antitrypsin deficiency is more often associated with lung and liver pathologies, α 1-antitrypsin deficient patients with concomitant IBD have been shown to develop more aggressive disease and rapid progression requiring surgery^[36]. In a study by Becker *et al.*^[37] it was found that individual fecal α 1-antitrypsin can predict prognosis in IBD patients.

Biomarker for GI allergic diseases

Eosinophil protein X: When lamina propria is damaged, eosinophils migrate into the gut lumen and multiple eosinophil granules related proteins are released, Table 2. These proteins contribute towards ongoing inflammation and tissue destruction associated with eosinophil related diseases like allergic diseases esophagitis, colitis, celiac disease, intestinal parasitic infections and IgE-mediated food allergy^[38]. There

is multiple eosinophil proteins including major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin and eosinophil peroxidase associated with eosinophilic activity during inflammation^[39,40]. This biomarker is however nonspecific and requires further studies to understand its role in eosinophil related disease pathology.

Biomarkers of gut health

Short-chain fatty acids: These are fatty acids with 1-6 carbon atoms, common ones are propionate, acetate, and butyrate produced as a results of metabolism of polysaccharides, oligosaccharides, peptides and glycoproteins by bacterial fermentation, absorbed by portal circulation and are an important energy source for colonic cells^[41,42]. They lower the gut pH by regulating fluid and electrolyte uptake *via* activation of apical Na^+/H^+ exchange receptor^[43]. These Short-chain fatty acids (SCFAs) are considered markers of colonic health and are known to have anti-inflammatory properties, Table 2. A study by Ohgashi *et al.*^[44], comparing colorectal carcinoma, adenoma and non-adenomatous subjects reported that compared to rest, subjects with carcinoma had decreased SCFA levels and altered microbial environment and pH.

Fecal secretory IgA: Immunoglobulin-A (IgA) are secreted by mucous membranes and as the name implies are antibodies important for mucosal immunity. These antibodies only form 15% for all immunoglobulins and in dimeric form called secretory IgA. This immunoglobulin forms a defense against enteric toxins and pathogenic organisms. Secretory IgA is mainly secreted in mucosal secretions like tears, saliva, sweat, genitourinary tract, GI tract, prostate and respiratory epithelium.

Fecal secretory IgA is a part of mucosal barrier against infections and is also known to inhibit inflammation playing a protective role; therefore it is considered as a marker of gut health^[45]. This biomarker is used to assess intestinal infections, coeliac disease and food allergies (Table 2)^[46,47]. Few studies have also evaluated its clinical utility as an alternate marker of IBD but its use for these diseases is limited due to non-specific nature of this molecule.

DISCUSSION

The currently used diagnostic tools for identifying GI diseases are endoscopic procedures. Endoscopies are costly, invasive, time consuming, and also require patient preparation. Most of the time endoscopic procedure also required sedation especially in pediatrics patients. Interpretation of an endoscopic report is also subjective and opinions of two experts can differ at time. Generally speaking non-invasive approaches like serological test, urinary, fecal or salivary biomarkers are logically more acceptable to patients. Fecal biomarkers are now increasingly being used and the development of

sensitive and specific immunochemical techniques have led to its increased utility.

Newer biomarkers with established diagnostic utilities in clinical use include lactoferrin, defensins and S100 proteins especially calprotectin. Calprotectin and lactoferrin are now also included in clinical practice guidelines in the management of IBD. However the clinical application of these biomarkers is well established for IBD but validation studies are still needed to understand their role in other GI pathologies. Also the reference cut offs used by each study is different, so there is need to standardize the assays and reference cutoffs of the established markers to clearly distinct diseased from non-diseased states. With more research to increase our understanding regarding roles of these biomarkers in GI health and disease, there is the potential for few more markers such as cathelicidins to be incorporated into clinical practice in near future.

Currently, apart from the fecal markers of inflammation there is not enough literature regarding fecal biomarkers clinical utility in other GI diseases or health. For example eosinophilic proteins have the potential to be used as disease markers for allergic states and parasitic infestations; very common in developing country. But these markers require more studies to better understand their roles in diseased states. Another advantage of these markers will be that they will provide more insight into the cause of disease. Furthermore as we are in an era of preventive medicine markers which can pick early changes in gut health are required so the patients screened out before developing a diseased state. In conclusion development of fecal biomarker and establishment of their clinical and diagnostic utilities is a developing field with a lot of promise, but we still need more research to validate these findings.

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Basic Study

Plecanatide-mediated activation of guanylate cyclase-C suppresses inflammation-induced colorectal carcinogenesis in $Apc^{+/Min-FCCC}$ mice

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Abstract

AIM

To evaluate the effect of orally administered plecanatide on colorectal dysplasia in $Apc^{+/Min-FCCC}$ mice with dextran sodium sulfate (DSS)-induced inflammation.

METHODS

Inflammation driven colorectal carcinogenesis was induced in $Apc^{+/Min-FCCC}$ mice by administering DSS in their drinking water. Mice were fed a diet supplemented with plecanatide (0-20 ppm) and its effect on the multiplicity of histopathologically confirmed polypoid,

flat and indeterminate dysplasia was evaluated. Plecanatide-mediated activation of guanylate cyclase-C (GC-C) signaling was assessed in colon tissues by measuring cyclic guanosine monophosphate (cGMP) by ELISA, protein kinase G-II and vasodilator stimulated phosphoprotein by immunoblotting. Ki-67, c-myc and cyclin D1 were used as markers of proliferation. Cellular levels and localization of β -catenin in colon tissues were assessed by immunoblotting and immunohistochemistry, respectively. Uroguanylin (UG) and GC-C transcript levels were measured by quantitative reverse transcription polymerase chain reaction (RT-PCR). A mouse cytokine array panel was used to detect cytokines in the supernatant of colon explant cultures.

RESULTS

Oral treatment of $Apc^{+/Min-FCCC}$ mice with plecanatide produced a statistically significant reduction in the formation of inflammation-driven polypoid, flat and indeterminate dysplasias. This anti-carcinogenic activity of plecanatide was accompanied by activation of cGMP/GC-C signaling mediated inhibition of Wnt/ β -catenin signaling and reduced proliferation. Plecanatide also decreased secretion of pro-inflammatory cytokines (IL-6, IL-1 TNF), chemokines (MIP-1, IP-10) and growth factors (GCSF and GM-CSF) from colon explants derived from mice with acute DSS-induced inflammation. The effect of plecanatide-mediated inhibition of inflammation/dysplasia on endogenous expression of UG and GC-C transcripts was measured in intestinal tissues. Although GC-C expression was not altered appreciably, a statistically significant increase in the level of UG transcripts was detected in the proximal small intestine and colon, potentially due to a reduction in intestinal inflammation and/or neoplasia. Taken together, these results suggest that reductions in endogenous UG, accompanied by dysregulation in GC-C signaling, may be an early event in inflammation-promoted colorectal neoplasia; an event that can potentially be ameliorated by prophylactic intervention with plecanatide.

CONCLUSION

This study provides the first evidence that orally administered plecanatide reduces the multiplicity of inflammation-driven colonic dysplasia in mice, demonstrating the utility for developing GC-C agonists as chemopreventive agents.

Key words: Guanylate cyclase-C; Uroguanylin; Plecanatide; Inflammation; Colorectal cancer

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Core tip: Plecanatide, an analog of human uroguanylin, binds and activates guanylate cyclase-C signaling to produce its anti-tumorigenic activity. This study provides the first evidence that oral treatment with plecanatide inhibits inflammation-driven colorectal carcinogenesis. The potential mechanism in $Apc^{+/Min-FCCC}$ mice appears to be agonist-mediated activation of guanylate cyclase-C signaling, resulting in inhibition of Wnt/ β -catenin pathway

and downregulation of pro-inflammatory cytokines and growth factors.

Chang WCL, Masih S, Thadi A, Patwa V, Joshi A, Cooper HS, Palejwala VA, Clapper ML, Shailubhai K. Plecanatide-mediated activation of guanylate cyclase-C suppresses inflammation-induced colorectal carcinogenesis in $Apc^{+/Min-FCCC}$ mice. *World J Gastrointest Pharmacol Ther* 2017; 8(1): 47-59 Available from: URL: <http://www.wjgnet.com/2150-5349/full/v8/i1/47.htm> DOI: <http://dx.doi.org/10.4292/wjgpt.v8.i1.47>

INTRODUCTION

Colorectal cancer (CRC) ranks third among newly diagnosed cancers in the United States and is the third most common cause of cancer mortality^[1]. Clinical studies indicate that patients with long-standing inflammatory bowel disease (IBD) have a 2 to 8-fold increased risk of developing CRC as compared to the general population^[2]. Although the precise etiology underlying inflammation-promoted CRC remains unclear, emerging data suggest that chronic inflammation, oxidative stress and excessive production of cytokines, chemokines and growth factors by infiltrating immune cells, may eventually lead to the development of dysplasia^[3-6]. Like sporadic CRC, development of IBD-promoted carcinogenesis follows a sequential progression of disease from low-grade to high-grade dysplasia, and eventually CRC^[7]. Prophylactic intervention with 5-aminosalicylate (5-ASA) is widely considered as a promising chemopreventive strategy^[8]. However, additional case-controlled studies with larger numbers of patients are needed to further elucidate the chemopreventive utility of 5-ASA in IBD-promoted CRC. Conceptually, chronic prophylactic intervention with an orally safe and mucosally active agent that not only suppresses inflammation but also regulates renewal of the gastrointestinal (GI) mucosa is desirable.

Uroguanylin (UG) and guanylin (GN), endogenous natriuretic peptides, are agonists of guanylate cyclase-C (GC-C) that are structurally similar to bacterial enterotoxin (ST), secreted by the pathogenic *Escherichia coli* (*E. coli*) responsible for traveler's diarrhea^[9]. Binding of these peptides to GC-C on the apical surface of epithelial cells lining the GI tract stimulates intracellular production of cyclic guanosine monophosphate (cGMP), resulting in activation of cGMP-dependent protein kinase G-II (PKG-II) and cystic fibrosis transmembrane conductance regulator. This leads to enhanced transepithelial efflux of Cl^- and HCO_3^- , inhibition of Na^+ absorption and passive secretion of water into the intestinal lumen; a process essential for a normal bowel movement^[10]. Thus, a major physiological function of UG and GN is to regulate ion and fluid homeostasis in the GI tract^[10-12].

GC-C signaling also plays a key physiological role in regulating the proliferative index of epithelial cells and maintaining the integrity of the GI mucosa^[10,13,14].

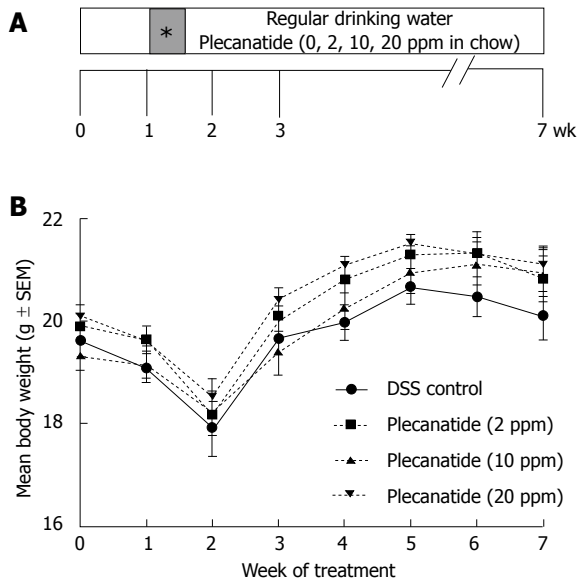


Figure 1 Inflammation-driven colorectal carcinogenesis in *Apc*^{+/Min-FCCC} mice. **A:** Outline depicting the experimental design of the animal study. Female *Apc*^{+/Min-FCCC} mice ($n = 23/\text{group}$) were randomized into four treatment groups: DSS alone (vehicle control) or DSS plus diet supplemented with 2, 10 or 20 ppm plecanatide. One week later, all animals were administered 2% DSS in the drinking water for 4 d (shaded box with asterisk) and regular water for the remainder of the study. At the time of euthanasia, (7 wk of study), the entire colon were fixed in formalin for histopathological evaluation; **B:** Body weights of *Apc*^{+/Min-FCCC} mice treated with either DSS alone or DSS plus a diet supplemented with indicated concentrations of plecanatide ($n = 20\text{--}23/\text{group}$). Body weights were obtained weekly, and DSS was administered to all animals on days 7–10 of study. DSS: Dextran sodium sulfate.

Thus, disruption of GC-C signaling, due to reduced production of UG and/or GN, could potentially lead to neoplastic transformation. Indeed, transcript levels of both UG and GN are reduced dramatically in colon polyps and adenocarcinomas^[13]. Furthermore, dietary supplementation of *Apc*^{+/Min} mice with human UG not only inhibits polyp formation but also delays tumor progression^[13]. Recent studies performed using GC-C and UG knockout mice further illustrate the involvement of GC-C signaling in the maintenance of homeostatic intestinal barrier function, gut permeability and intestinal epithelial cell proliferation^[15]. Recently, we demonstrated that oral treatment with GC-C agonists such as plecanatide or dolcanatide effectively ameliorated GI inflammation in acute and chronic models of experimental colitis in murine models^[16]. Thus, treatment with UG to overcome this deficiency may represent a promising approach for the prevention of inflammation-driven CRC.

The present study provides the first evidence to demonstrate that oral treatment with plecanatide effectively suppresses the formation of inflammation-driven CRC in *Apc*^{+/Min-FCCC} mice.

MATERIALS AND METHODS

Materials

DSS (molecular weight 30000–40000) was purchased from MP Biomedicals (Solon, OH). All other chemicals

and reagents were obtained from commercial vendors. Plecanatide (H-Asn¹-Asp²-Glu³-Cys⁴-Glu⁵-Leu⁶-Cys⁷-Val⁸-Asn⁹-Val¹⁰-Ala¹¹-Cys¹²-Thr¹³-Gly¹⁴-Cys¹⁵-Leu¹⁶-OH; Disulfide bond between Cys⁴ and Cys¹²; Cys⁷ and Cys¹⁵) was synthesized for this study according to procedures described previously^[17].

Animals

Female *Apc*^{+/Min-FCCC} (C57BL/6J) mice (7-wk-old, $n = 92$) were obtained from the Laboratory Animal Facility at Fox Chase Cancer Center (FCCC)^[18]. Mice were genotyped for a point mutation in codon 850 of the *Apc* gene^[19]. Animals were maintained in a temperature- and humidity-controlled room and received Teklad Global 2018SX diet (Harlan Teklad, Madison, WI) and drinking water *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee at FCCC (IACUC # 08-4). Drug-supplemented diet was prepared as described previously^[13].

Methods

Experimental design: Inflammation-promoted colorectal neoplasia was induced by administering 2% dextran sodium sulfate (DSS) to female *Apc*^{+/Min-FCCC} mice in the drinking water as outlined in Figure 1A. At treatment week 0 (8 wk of age), mice were randomized to one of four experimental groups: DSS treatment alone (no plecanatide treatment (control)) or DSS plus 2, 10 or 20 ppm plecanatide in the diet ($n = 23/\text{group}$). All animals were administered DSS for 4 d beginning at treatment week 1 and received regular water for the remainder of the study (Figure 1A). At the end of the study, the entire small intestine and colon were excised, cut longitudinally and rinsed with saline. An equivalent strip of the small intestine and colon from each animal was snap frozen for molecular analysis of UG and GC-C transcript levels by quantitative reverse-transcription polymerase chain reaction (RT-PCR). The remainder of the colon was fixed in 10% formalin overnight, cross-sectioned at 2 mm intervals and processed for histopathological review.

Histopathological analyses: Sections stained with H and E were histopathologically evaluated for neoplasia in a blinded manner, as described previously^[20]. All classifications were based on standardized morphology and nomenclature for the human pathology of inflammation-promoted colorectal neoplasia^[21]. A diagnosis of carcinoma was assigned when neoplastic glands had invaded into the muscularis mucosae or beyond. Any dysplasia or cancer exhibiting an elevated growth pattern was considered polypoid. Non-polypoid (flat) lesions were elevated less than 2-fold above the adjacent non-neoplastic colorectal mucosa. Lesions that could not be readily classified as either polypoid or non-polypoid were categorized as indeterminate.

Immunohistochemistry: Ki-67 was selected as a biomarker of cell proliferation. Antigen retrieval was

performed prior to staining in a Ventana Benchmark XT automated stainer (Tucson, AZ). All buffers and washes were per standard XT protocol. For Ki-67 staining, sections were incubated with Ki-67 primary antibody (1:1500 dilution; Vector Laboratories, Inc., Burlingame, CA) for one hour at room temperature. Negative controls were processed with rabbit IgG at approximately the same protein concentration as the primary antibody. Staining was detected using a rabbit secondary antibody kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. All sections were counterstained with lite hematoxylin. Only cells with nuclear staining of Ki-67 were considered positive. The number of Ki-67 positive cells in dysplasias (2 fields/dysplasia, 600 ×) and non-neoplastic colonic crypts (20 crypt columns/animal, 600 ×) were counted and recorded as a Ki-67 labeling index (number of positive cells/total number of cells evaluated). The rate of proliferation of each tumor was established and the mean rate of all tumors in the treatment group was calculated.

The cellular localization of β -catenin was determined using specific polyclonal antibodies (1:4000 dilution; Sigma, St Louis, MO). Negative controls were processed with rabbit IgG. Staining was detected using a goat anti-rabbit secondary antibody kit (Vector Laboratories, Inc.) as per the manufacturer's instructions. Sections were counterstained with lite hematoxylin. The number of tumor cells with nuclear localization of β -catenin was counted and expressed as a percentage of the total number of tumor cells per field (400 ×).

Immunoblotting: One centimeter long colon tissues from 6 animals per group were pooled and homogenized in RIPA buffer (10 mmol/L Tris, pH 7.2; 150 mmol/L NaCl, 1% sodium deoxycholate, 1% triton × 100, 0.1% SDS, 0.1 mmol/L Na_3VO_4 ; 50 mmol/L NaF), containing a protease inhibitor cocktail (Boehringer Mannheim, GmbH, Germany). The homogenate was centrifuged at 12000 g for 15 min at 4 °C and the supernatant was used as crude tissue lysate. The crude lysates (approximately 50 g protein) were subjected to 10% SDS-PAGE under reducing conditions, followed by immunoblotting with antibodies specific for β -catenin (1:4000 dilution, Sigma), PKG-II (1:200, dilution Santa Cruz Biotechnology, Inc., CA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10000 dilution, Ambion), GC-C (1:500, Santa Cruz Biotechnology Inc., CA), phospho-VASP and c-myc (1:1000, Cell Signaling, MA, United States), cyclin D1 (1:1000, Abcam, MA, United States), and β -actin (1:1000, Chemicon, CA). Blots were developed using the ECL plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom) or LiCor blotting system. The resulting images were analyzed using the FluorChem E system (Cell Biosciences, Santa Clara, CA).

Measurement of cyclic GMP in tissue lysates: Colon tissues (1 cm) from 6 animals per group were pooled

to prepare crude tissue lysates. The levels of cGMP were determined using an ELISA kit (Cayman Chemical Co., Ann Arbor, MI)^[13]. The protein concentration of the lysates was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Results were normalized per mg protein and expressed as mean pmol \pm SEM.

Quantification of UG and GC-C transcript levels by RT-PCR:

Representative areas of the proximal and distal small intestine and colon were randomly selected from DSS control and DSS + plecanatide-treated mice ($n = 4$ -5/group) for analysis. Total RNA was extracted from 5-10 mg of tissue using the RNA-queous[®]-4PCR Kit (Ambion, Austin, TX) and quantified using a Nanodrop2000 (Thermo Fisher, Waltham, MA). Total RNA (1 μ g) was reverse-transcribed to cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. Quantitative RT-PCR amplification and analysis were carried out using a LightCycler480 (Roche, Basel, Switzerland), UG and GC-C specific TaqMan reagents (Integrated DNA Technologies, Coralville, IA) and RT-PCR Master Mix (Roche, Basel, Switzerland). All amplification reactions (20 L total volume) were performed in duplicate with 10 ng cDNA (based on input RNA) and subjected to 35 PCR cycles using parameters set by the manufacturer. GAPDH was used as an endogenous control. The data generated were analyzed and expressed as target gene expression relative to endogenous control, using the comparative Ct method and the $2^{-\Delta\Delta\text{Ct}}$ formula. Results are expressed as fold change in relative levels of UG or GC-C transcripts per segment of small intestine or colon of plecanatide-treated mice, as compared to those of control mice treated with only DSS.

DSS treatment to induce acute inflammation in $\text{Apc}^{+/Min-FCCC}$ mice:

An independent study was designed to evaluate the effect of plecanatide on GC-C signaling and cytokine expression during the acute phase of colonic inflammation. $\text{Apc}^{+/Min-FCCC}$ mice ($n = 12$; 6-8 wk old) were administered 2% DSS in the drinking water for 4 d followed by 3 d of regular water. Starting on day 1, six animals received an oral gavage of plecanatide (2.5 mg/kg body weight) daily while the other six were administered vehicle (0.9% sodium chloride solution, Sigma, St. Louis, MO). Vehicle control animals ($n = 6$) received regular drinking water (no DSS) and were administered an oral gavage of saline daily. It should be noted that the amount of plecanatide delivered by a single oral gavage at this dose is similar to that ingested daily when animals were administered a diet supplemented with 10 ppm plecanatide in the main tumorigenesis study. Mice were euthanized on day 7, and the entire colon was excised. Part of the colon tissue from each animal was snap frozen for analysis of intracellular cGMP and determination of the expression of GC-C, PKG-II, p-VASP and β -actin by immunoblot.

The remaining tissue was used immediately for explant cultures as described below.

Explant culture: Tissues were washed in PBS containing 100 units of penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B per milliliter (1 x antibiotic and anti-mycotic solution; Sigma, St. Louis, MO) and cut into 1 cm pieces. Tissue explants were cultured in a 24-well plate overnight in RPMI 1640 media (Mediatech, Manassas, VA) in the absence or presence of 1 µmol/L plecanatide at 37 °C in a CO₂ incubator. After the incubation period, explants were snap frozen and stored for analysis of cyclin D1, c-myc and β-actin expression by immunoblot.

Cytokine analysis: Expression of select mouse cytokines was detected in the spent media of pooled explant cultures ($n = 6/\text{pool}$) using a mouse Proteome Profiler Panel A kit array panel (R and D Systems, Minneapolis, MN). The mean intensities of the dot blots were calculated using ImageJ software (NIH).

Statistical analysis

Wilcoxon 2-sample test and analysis of variance (ANOVA) were used to compare differences between groups such as body weight, lesion multiplicity, and lesion type. Student's *t* test was used to evaluate differences in cGMP, Ki-67 labeling index, expression of total and nuclear β-catenin, cytokines, PKG-II protein, UG and GC-C transcript levels.

RESULTS

Plecanatide reduced inflammation-promoted dysplasia in Apc^{+/Min-FCCC} mice

All experimental treatments were well tolerated. The rate of survival was: 91% for mice in the control group, 83% for mice administered 2 ppm plecanatide, and 96% for mice receiving diet supplemented with either 10 or 20 ppm plecanatide. As observed previously in this model^[22], body weights declined immediately following DSS treatment and increased gradually thereafter in all groups (Figure 1B). The body weights of mice treated with DSS + plecanatide increased more rapidly than did those of mice receiving DSS + vehicle. Tissues were also scored for the degree of inflammation (data not shown). Consistent with our previous experience in this animal model, the inflammation scores were generally low, most likely due to anticipated resolution of the colonic inflammation by the end of the study.

The effect of plecanatide on the multiplicity of polypoid, flat, indeterminate and total colonic dysplasias was determined (Figure 2). While the multiplicity of all morphological subtypes of colon lesions in mice treated with 2 ppm plecanatide did not differ from that of controls, a reduction in the multiplicity of each of the subtypes of dysplasia was observed in mice treated with higher doses of the drug. For example, 10 and 20 ppm

plecanatide reduced polypoid dysplasias (Figure 2A), with a statistically significant reduction (approximately 40%) observed at a dose of 20 ppm, as compared to the control group ($P = 0.05$). A similar reduction in the multiplicity of non-polypoid/flat ($P = 0.041$) and indeterminate ($P = 0.05$) dysplasias was observed post treatment, but only in mice receiving 10 ppm plecanatide (Figure 2B and C). Surprisingly, the higher dose of plecanatide (20 ppm) did not produce an appreciable reduction in the multiplicity of either flat or indeterminate colonic dysplasias. A statistically significant reduction ($P = 0.028$) in total colonic dysplasia was observed in mice administered 10 ppm of plecanatide in the diet (Figure 2D). As previously observed with oral UG treatment^[13], plecanatide also reduced the multiplicity of small intestinal tumors (data not shown). Since suppression of polyp formation by oral treatment with GC-C agonists has now been well-established^[13,23], this study focused only the effect of orally administered plecanatide on the multiplicity of colonic dysplasias. Therefore, subsequent analyses were performed on colon tissues from mice treated with 10 ppm plecanatide.

Plecanatide mediated activation of GC-C signaling

Although UG, GN, and other GC-C related agonists are known to stimulate production of cGMP via activation of GC-C, in epithelial cells lining the GI tract, and cultured T84 and Caco-2 cells^[12,24,25], it was of interest to evaluate if orally administered plecanatide could stimulate cGMP production within the murine colon. Acute inflammation was induced in mice as described in the Materials and Methods section. Colon tissues (1 cm piece) from 6 mice within a treatment group were pooled, homogenized, and crude lysates were used to measure cGMP. A significant reduction in the cGMP levels was observed in colon tissues from DSS-treated animals as compared to vehicle controls ($P = 0.01$). Oral treatment with plecanatide (2.5 mg/kg) completely restored the DSS-mediated reduction in cGMP levels (Figure 3A).

PKG-II, a cGMP-dependent protein kinase, is activated by cGMP following activation of GC-C by its agonists. PKG-II is expressed on epithelial cells lining the GI tract and undergoes auto-phosphorylation upon activation of GC-C signaling^[26]. Colon tissues from mice with DSS-induced inflammation were homogenized and lysates were subjected to immunoblotting with antibodies specific for phosphorylated-vasodilator-stimulated phosphoprotein (p-VASP), PKG-II and GC-C (Figure 3B). The p-VASP antibody detects only Ser²³⁹ phosphorylated VASP. Activated PKG-II expression, seen on blots as a partially resolved doublet, was considerably higher in colon tissues from mice treated with both DSS and plecanatide as compared to those treated with only DSS. The level of p-VASP was also much higher in colon tissues from DSS + plecanatide treated mice as compared to that observed in tissues from DSS treated mice. Colonic expression of GC-C was comparable among all

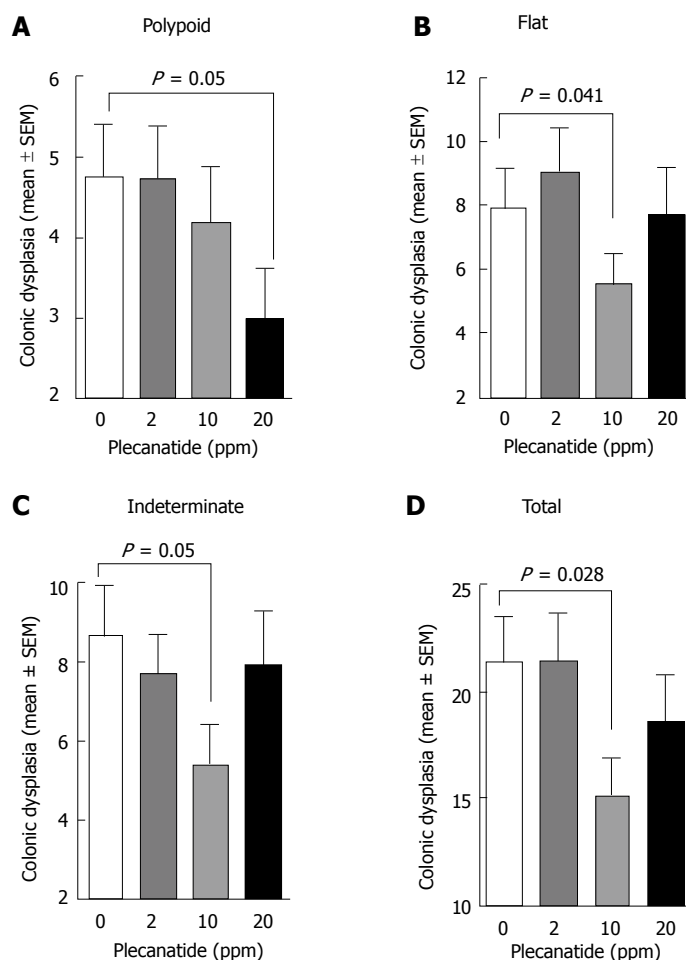


Figure 2 Treatment with plecanatide inhibits inflammation-associated colonic dysplasia in dextran sodium sulfate-treated *Apc*^{+/Min-FCCC} mice. Analyses revealed the number of pathologically confirmed polypoid (A), flat (B), indeterminate (C) and total (D) dysplasias within the colon of DSS-treated mice following administration (7 wk) of either control diet or diet supplemented with varying doses of plecanatide ($n = 23/\text{group}$). Wilcoxon 2-sample test and analysis of variance (ANOVA) were used to compare the multiplicity of dysplasias in independent groups. A P value ≤ 0.05 was considered statistically significant. DSS: Dextran sodium sulfate

cohorts, irrespective of the treatment group (Figure 3B). Collectively, these results suggest that GC-C signaling is activated in the colon by orally administered plecanatide.

Plecanatide reduces proliferation of colonic epithelial cells

Immunohistochemical staining of Ki-67 was performed using non-neoplastic (normal) and neoplastic mucosa from the colons of mice treated with DSS or DSS + plecanatide (10 ppm). As depicted in Figure 4A, plecanatide reduced the number of Ki-67 positive epithelial cells in both the non-neoplastic and neoplastic mucosa. However, the reduction in Ki-67 labeling only achieved significance in the neoplastic mucosa ($P < 0.001$). Similarly, although the number of caspase-3 positive cells was increased in both non-neoplastic and neoplastic colon tissue, the elevation was significant ($P = 0.05$) only in plecanatide treated non-neoplastic tissue (data not shown). It should be noted that endogenous UG expression and GC-C signaling-mediated regulation of epithelial cell homeostasis is not altered in the normal epithelium. In addition, the rate of cell proliferation is also much higher in tumor tissue as compared to the normal epithelium. Thus, it is possible that oral treatment with plecanatide has a more pronounced inhibitory effect on proliferation in tumor tissue than in the normal epithelium. To further confirm the anti-

proliferative activity, crude lysates of colon tissues from mice treated with vehicle, DSS and DSS + plecanatide (2.5 mg/kg) were examined by immunoblotting with antibodies specific for c-myc and cyclin D1 (markers of proliferation) (Figure 4B). Plecanatide reduced levels of c-myc and phosphorylated cyclin D1 (slower moving band) in colon tissues as compared to treatment with either vehicle or DSS alone. Taken together, these results suggest that plecanatide reduces proliferation of epithelial cells lining the GI mucosa.

A statistically significant reduction in the levels of total β -catenin was observed in colon tissues from mice administered 10 ppm plecanatide in the diet as compared to control DSS-treated mice. The densitometry of the blot is shown in Figure 5A. Consistent with our previous report^[18], immunohistochemical staining revealed membranous localization of β -catenin within the non-neoplastic colonic mucosa. Strong cytoplasmic and nuclear staining of β -catenin staining was observed in colonic dysplasias from DSS-treated mice (panel I, Figure 5B). Treatment with plecanatide (10 ppm) reduced nuclear staining of β -catenin, with a concomitant increase in its localization to the membrane of neoplastic cells (Figure 5B panel II). The densitometric analysis revealed that plecanatide treatment reduced β -catenin levels in the nucleus by 41% ($P = 0.02$) as compared to analogous neoplastic regions within the

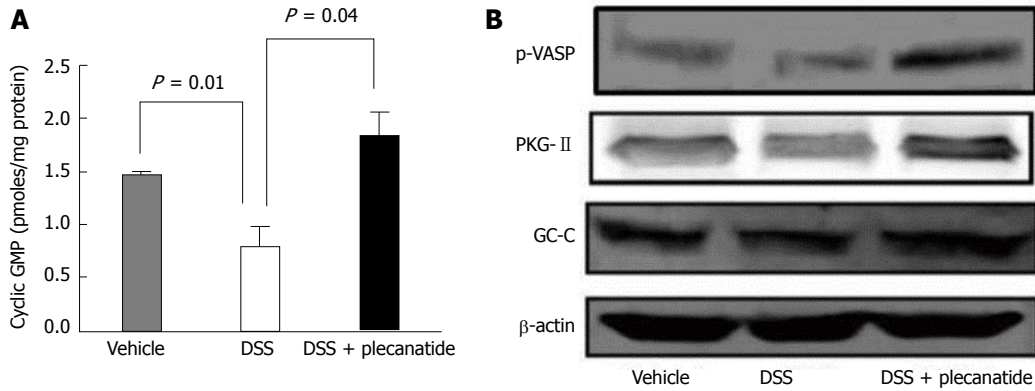


Figure 3 Orally administered plecanatide activates guanylate cyclase-C signaling within the colon. Effect of plecanatide on (A) stimulation of cGMP production and (B) expression of phosphorylated VASP, PKG-II and GC-C in colon tissues from $Apc^{+/Min-FCCC}$ mice with DSS-induced acute inflammation ($n = 6/\text{group}$). Mice with acute inflammation were administered plecanatide (2.5 mg/kg) by oral gavage; a dose equivalent to that ingested daily by animals fed a diet supplemented with 10 ppm plecanatide in the main tumorigenesis study. Colon tissue (1 cm) from 6 animals per group was pooled to prepare cell lysates. Intracellular cGMP levels depicted in (A) are expressed as pmoles/mg protein \pm SEM. Student *t* test was used to evaluate differences in cGMP between treatment groups. *P* values ≤ 0.05 were considered statistically significant. Representative Western blot analyses of phospho-VASP, PKG-II and GC-C were performed using appropriate antibodies. To demonstrate equivalent protein loading for each condition, membranes were probed subsequently with β -actin antibody. GC-C: Guanylate cyclase-C; GMP: Guanosine monophosphate; p-VASP: Phospho-vasodilator-stimulated phosphoprotein; PKG- II : Protein kinase G-II.

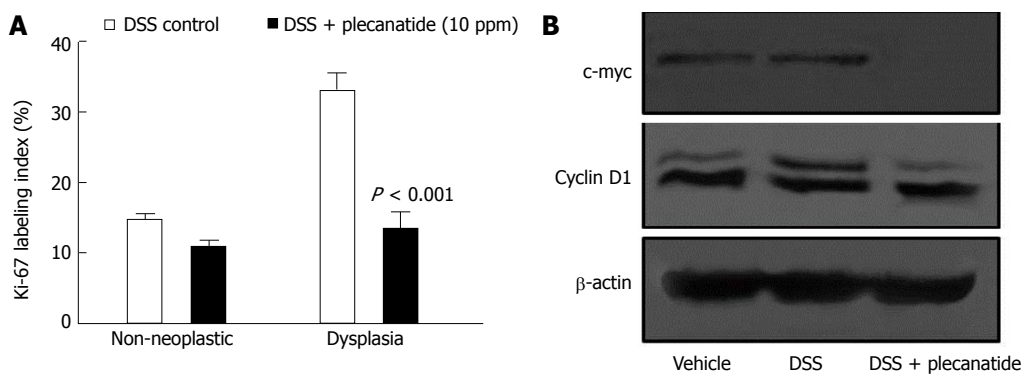


Figure 4 Effect of plecanatide on markers of proliferation in colonic epithelial cells from $Apc^{+/Min-FCCC}$ mice with dextran sodium sulfate-induced inflammation. A: Normal (non-neoplastic) and neoplastic colon tissues from mice treated with DSS only and DSS + plecanatide (10 ppm) were stained with antibodies specific for Ki-67. Nuclear staining of Ki-67 (positive) was recorded as a labeling index (number of positive cells/total number of cells evaluated; mean \pm SEM). Statistical comparisons between DSS control and DSS plus plecanatide-treated groups ($n = 7-9$ mice per group) were performed using the Student *t* test. A *P* value ≤ 0.05 was considered significant; B: Colon tissue (1 cm) from 6 animals per group was pooled to prepare cell lysates. A representative immunoblot demonstrating the effect of plecanatide on expression of c-Myc and cyclin D1 is shown. β -actin was used to normalize protein loading. DSS: Dextran sodium sulfate.

colons of DSS-treated mice (Figure 5B, panel III).

Plecanatide treatment restores UG expression

Consistent with previous reports that expression of UG is reduced dramatically in inflamed tissues from colitic mice and in colon biopsies from IBD patients^[13,27], UG transcript levels were also reduced significantly in intestinal tissues following DSS treatment as compared to those of vehicle-treated mice (data not shown). Since administration of plecanatide reduces GI inflammation^[16] and the multiplicity of colonic dysplasias in colitic mice, it was important to determine if plecanatide treatment increased transcript levels of UG and GC-C following amelioration of GI inflammation in intestinal tissues from $Apc^{+/Min-FCCC}$ mice. UG and GC-C expression was determined in the proximal and distal segments of the small intestine and colon by quantitative RT-PCR (Figure 6A). UG expression was increased significantly within the

proximal small intestine and proximal colon of animals receiving oral plecanatide. A similar increase in UG expression was observed in the distal small intestine, but did not achieve statistical significance. It should be noted that the relative expression of UG is known to be extremely low in the distal colon as compared to that in the proximal region of the small intestine^[24]. Thus, accurate quantitative measurement of UG expression in the distal colon segment may be compromised by low endogenous levels. No appreciable change in relative levels of GC-C transcripts was observed in either the small intestine or colon following plecanatide treatment (Figure 6B).

Plecanatide downregulates pro-inflammatory cytokines in colon explants

Induction of colonic inflammation with DSS is known to increase the production of proinflammatory cytokines

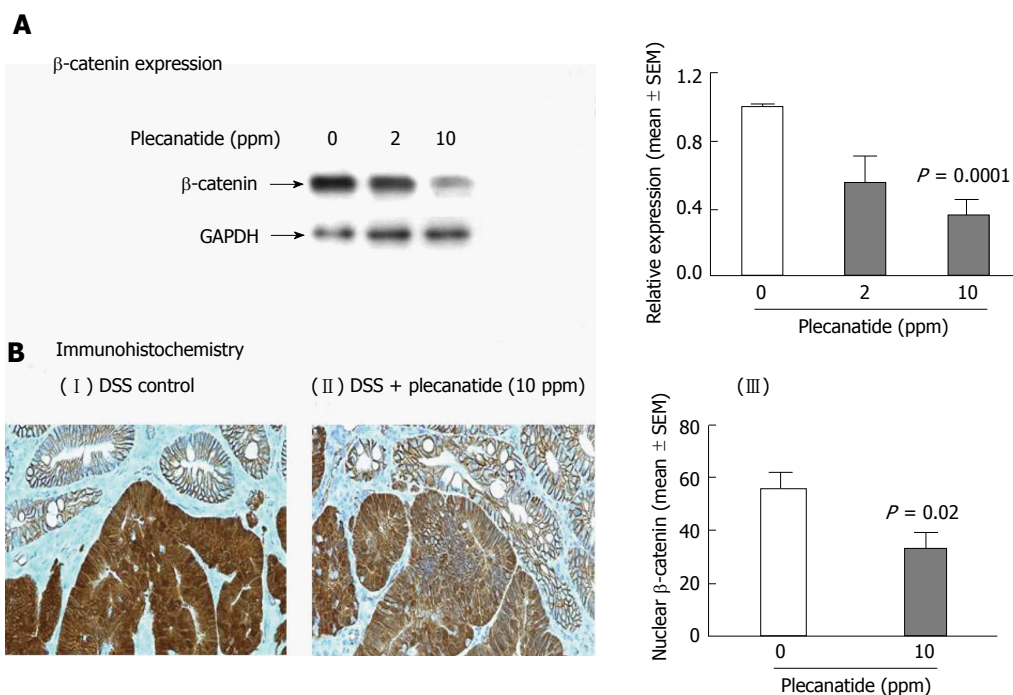


Figure 5 Total β -catenin expression is reduced within the colon of $Apc^{+/Min-FCC}$ mice treated with dextran sodium sulfate plus plecanatide. A: Western blot analysis and the associated densitometric quantification of levels of total β -catenin expression (mean \pm SEM) within the colon; B: Immunohistochemical localization of β -catenin within the colonic mucosa. Membranous localization of β -catenin was observed within the normal colonic mucosa irrespective of the treatment group, while cytoplasmic and nuclear β -catenin staining predominant in adenomas from DSS-treated mice (panel I). Plecanatide treatment caused a significant reduction in nuclear staining of β -catenin in dysplasias, while the cell membranes exhibited enhanced protein localization (panel II). The number of tumor cells with nuclear localization of β -catenin was counted in distal colon tumors ($n = 7-9$ mice/group) and expressed as a percentage of the total number of tumor cells per 400 X field (panel III). Statistical comparisons between DSS control and DSS plus plecanatide-treated groups were performed using the Student's *t* test. A *P* value of ≤ 0.05 was considered significant. DSS: Dextran sodium sulfate; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; .

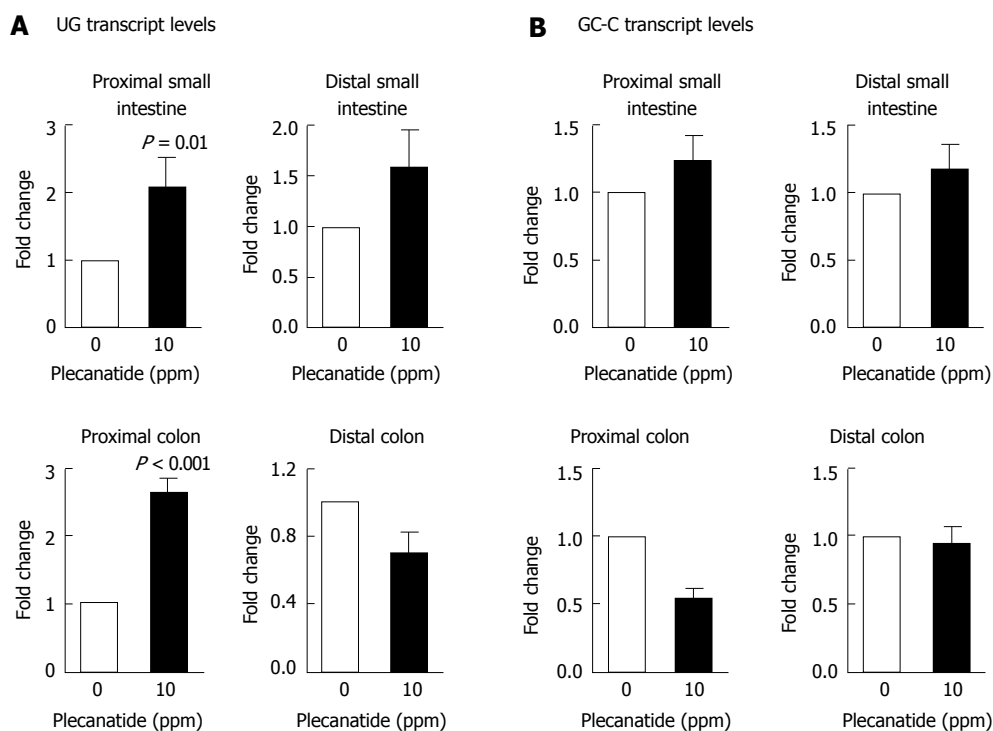


Figure 6 Effect of plecanatide on expression of uroguanylin and guanylate cyclase-C. The relative levels of UG (A) and GC-C (B) transcripts in the small intestine and colon of DSS-treated and DSS + plecanatide treated $Apc^{+/Min-FCC}$ mice ($n = 5-6$ /group) were determined by quantitative RT-PCR and normalized to those of GAPDH in the same sample. Transcript levels are expressed as fold change (mean \pm SEM) as compared to control samples treated with only DSS. Student's *t*-test was used to evaluate statistical differences between DSS control and plecanatide-treated mice. A *P* value of ≤ 0.05 was considered significant. GC-C: Guanylate cyclase-C; UG: Uroguanylin; DSS: Dextran sodium sulfate.

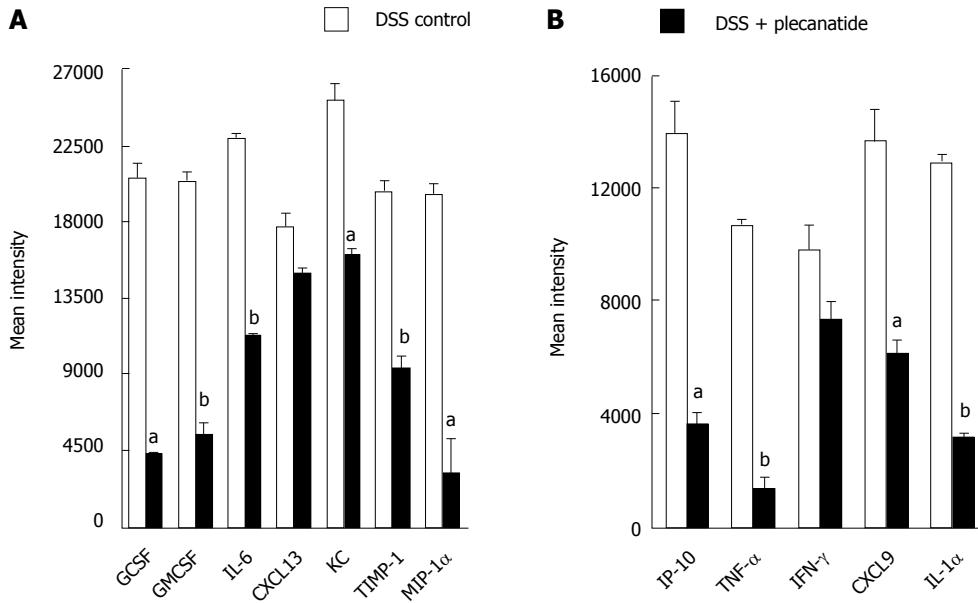


Figure 7 Effect of plecanatide on cytokine expression. Expression of cytokines, chemokines and growth factors was measured in the spent media of colonic explant cultures derived from mice treated with DSS only or DSS + plecanatide. Murine cytokines, chemokines and growth factors were measured in pooled supernatants ($n = 6/\text{pool}$) using membranes coated with specific capture antibodies (Proteome Profiler Panel A kit; R and D Systems, Minneapolis, MN). Immunoblots were scanned using Image J software. The data represent the mean intensity of cytokine/chemokine levels in colonic samples derived from untreated and plecanatide-treated mice. Student's t test was used to evaluate statistical differences in the secretion of cytokines/chemokines between DSS control and DSS + plecanatide treated samples. ^a P value ≤ 0.05 , ^b $P \leq 0.01$; vs DSS control.

and growth factors in mice^[28]. Thus the effect of plecanatide treatment on activation of GC-C signaling and on secretion of select pro-inflammatory cytokines, chemokines and growth factors was examined in colon explants from $\text{Apc}^{+/Min-FCCC}$ mice with acute inflammation. Consistent with the *in vivo* results shown in Figure 3, plecanatide treatment activated GC-C signaling, increased cGMP production and restored levels of PKG-II in colon explants (data not shown), confirming that orally administered plecanatide is able to activate GC-C signaling within the colon. Next, we determined the effect of plecanatide treatment on the secretion of cytokines and growth factors in explant cultures of colon tissues from mice in the above study. Analysis of the supernatant from pooled explant cultures using a mouse cytokine array revealed a significant reduction in secretion of pro-inflammatory cytokines (IL-1 α , IL-6 and TNF α), chemokines (IP-10, MIP-1 α , KC and CXCL9) and growth factors (GCSF and GMCSF) in DSS + plecanatide treated mice as compared to mice treated with DSS alone (Figure 7). These data are consistent with the anti-inflammatory activity of plecanatide in experimental models of murine colitis^[16].

DISCUSSION

This is the first study to demonstrate that oral treatment with plecanatide reduces the multiplicity of DSS-promoted colonic dysplasias in mice; a well-established model for studying inflammation-associated colorectal carcinogenesis^[29]. Results presented here demonstrate that $\text{Apc}^{+/Min-FCCC}$ mice treated with DSS exhibit an

increased multiplicity of colonic dysplasias and that oral treatment with plecanatide produces a statistically significant reduction in inflammation-induced dysplasia in these mice. Although the multiplicity of all morphological subtypes of colonic dysplasias was reduced in mice exposed to plecanatide, the dose required to achieve tumor inhibition varied. For example, plecanatide reduced the formation of polypoid dysplasias in a dose-dependent manner, with an approximate 40% reduction observed at 20 ppm. On the other hand, a similar magnitude of reduction in the multiplicity of flat and indeterminate colonic dysplasias was observed at 10 ppm, with no appreciable inhibition recorded at a higher dose (20 ppm). The chemopreventive effect of NSAIDs and cyclooxygenase-2 inhibitors against inflammation-driven colorectal carcinogenesis has been evaluated in several animal models including $\text{Apc}^{+/Min}$ mice. While the reduction in colonic tumors in these studies was approximately 50%^[29], it is noteworthy that the observed approximately 40% reduction in dysplasia following plecanatide treatment, is comparable to the result obtained from a meta-analysis of 5-ASA in a clinical setting (37%-49% reduction in neoplasia)^[30]. Our results, indicating a differential response of lesion types to plecanatide, are consistent with previous observations suggesting that flat and polypoid dysplasias arise *via* distinct genetic mechanisms^[31,32] and respond differently to prophylactic therapies^[8]. In addition, a differential response of polypoid and flat dysplasias to celecoxib (Celebrex[®]) has also been observed^[32].

The lack of an observed plecanatide dose response

is consistent with the results of several animal studies examining the effect of plecanatide and dolcanatide (another UG analog) on amelioration of colitis in mice^[16]. Orally administered plecanatide or dolcanatide activates GC-C receptors and produces fluid distention only in the duodenum and jejunum^[33], suggesting that they act primarily in the proximal intestine to stimulate fluid secretion. Therefore, a higher dose of plecanatide may lead to excess fluid production in the proximal intestine, resulting in dilution of the orally administered plecanatide prior to reaching the colon segments. It should also be noted that plecanatide acts locally by binding to GC-C expressed on epithelial cells lining the luminal surface of the GI tract and its systemic absorption is not needed to produce a pharmacological effect. Thus, lack of a dose response could also be due to saturation of the GC-C that is available on the luminal surface of the GI mucosa. In addition, results from animal studies conducted by this group with orally administered 5-ASA demonstrate that higher doses do not confer greater protection from the formation of colitis-associated tumors^[8]. Thus, development and optimization of a new formulation of plecanatide that bypasses the proximal intestine is warranted to not only decrease fluid secretion, but also enhance its chemoprotective activity. A longer duration of exposure to plecanatide would also allow more time for the colonic mucosa to heal and could potentially enhance chemopreventive response to treatment.

Activation of GC-C signaling by its ligands is associated with an increase in cGMP, a decrease in cyclin D1, delayed cell cycle progression and reduced DNA synthesis^[34-36]. Importantly, oral administration of cGMP restored crypt proliferative homeostasis and reduced proliferation (Ki-67 positive cells) in the crypts, but not in the villi of *Gucy2c*^{-/-} mice^[36]. In addition, normal functioning of GC-C signaling appears to also regulate the balance between proliferation and differentiation in the intestinal epithelium^[37]. In this context, GC-C signaling plays a key role in organizing the crypt-surface axis, restricting the depth of the crypts and the number of proliferating cells and regulating the rate of cell cycle progression through the G1-S transition^[23]. Consistent with these findings, UG and *E. coli* ST inhibit the proliferation of T84 and Caco-2 colon carcinoma cells^[13,14]. Data from the present study demonstrating that plecanatide reduced levels of β -catenin, c-myc and cyclin D1 as well as nuclear and total β -catenin provide further support for these findings. Of relevance, treatment with dolcanatide also reduced transcript levels of c-myc, cyclin D1 and Birc5 (survivin) in T84 cells^[38]. This study suggests that orally administered plecanatide may act *via* cGMP/GC-C signaling to mediate downregulation of Wnt/ β -catenin signaling within the colon.

The ability of plecanatide to retard the progression of inflammation-associated colorectal neoplasia represents an extension of our prior findings, demonstrating that oral treatment with UG suppressed polyp formation

in *Apc*^{+/-Min} mice^[13] and plecanatide ameliorated colitis in mice^[16]. It should be noted that the expression of UG is reduced significantly in inflamed tissue from IBD patients as well as in colon tissue from mice with colitis^[13,27,39]. These findings suggest that loss of UG expression, in the presence of key mutations in the *APC* gene, results in dysregulation of GC-C signaling and in downstream activation of the Wnt/ β -catenin pathway. The resulting transactivation of genes responsible for hyperproliferation and anti-apoptotic mechanisms may be the quintessential events during the early stages of neoplastic transformation in colonocytes. In this context, silencing of GC-C signaling is typically associated with early loss of APC heterozygosity and subsequent AKT-mediated inhibition of apoptosis; a potential trigger for neoplastic transformation in colonocytes^[23,36]. These reports also suggest that loss of GC-C signaling could be associated with increased susceptibility to intestinal carcinogenesis in mice. However, the possibility that the observed reduction in polyp formation occurs *via* a non-GC-C mechanism^[40] cannot be completely ruled out. Transcription of the GC-C gene can be regulated by β -catenin/TCF signaling^[40]. Interestingly, treatment with *E. coli* ST peptide stimulated duodenal HCO₃⁻ secretion, albeit at a reduced level, in GC-C^{-/-} mice^[41], suggesting the existence of a non-GC-C mechanism, possibly involving a UG/ST receptor yet to be identified.

A known human kindred mutation that causes altered expression of GC-C and presents clinically as bowel dysfunction in Norwegian families has been reported^[42]. This GC-C "gain-of-function" mutation in infants of these families leads to chronic diarrheal diseases, often accompanied by electrolyte imbalance, dehydration, metabolic acidosis and ileal inflammation. Two additional kindred mutations in *GUCY2C* were reported in two unrelated Bedouin families, where the "loss-of-function" of GC-C was associated with meconium ileus^[43]. Although there is no mention of an association of these kindred mutations with increased susceptibility to colon cancer, deregulated GC-C signaling early in life may be the key event that increases susceptibility to intestinal inflammation and eventually colon cancer.

The precise cause for downregulation of UG and GN and early dysregulation of GC-C signaling during inflammation and colon carcinogenesis remains largely unexplored. Nevertheless, it is known that the genes encoding endogenous GC-C ligands UG and GN are located on chromosome 4 in mice and 1p34-35 in humans, a region lost frequently during human colon carcinogenesis^[44-46]. Our results, albeit preliminary, suggest that the level of UG transcripts in the small intestine and proximal colon increase following treatment with plecanatide. One possible explanation for the restoration of UG expression is the mucosal healing that eventually follows inhibition of inflammation and/or colorectal dysplasia. It should be noted that UG transcripts levels were measured in intestinal tissue samples comprised of both neoplastic and adjacent

normal tissue. Since the expression of UG is lost in colon polyps and tumors but not in the surrounding normal colonic mucosa^[13], measurement of UG and GC-C transcript levels could be influenced by the number of tumors present, the severity of inflammation, and the proportion of normal tissue in the sample. A more accurate comparative analysis of UG and GC-C expression in microdissected inflamed, normal vs tumor tissue will be needed in the future. It is known that UG is predominantly expressed in the small intestine and proximal colon, whereas GN expression is more abundant in the colon^[24]. An analysis of GN transcript levels in colon tissue would be useful to better understand the cooperative functions of UG and GN within the colon. Another limitation in this study is that expression of UG was examined only at the transcriptional level and not at the protein level. Antibodies specific for UG are being generated currently for immunohistochemical analysis of UG expression in normal, inflamed and tumor tissue; studies that are anticipated to provide new insight into the molecular basis for the disruption of GC-C signaling during colon carcinogenesis.

In summary, results from the present study suggest that administering plecanatide to overcome a deficiency in endogenous GC-C ligands ameliorates inflammation/colitis and delays progression to CRC. These findings represent a new role for GC-C agonists in the prevention of inflammation-associated CRC in humans. Recent clinical studies suggest that plecanatide is a safe and orally active drug candidate, with promising potential for use in the treatment of various GI disorders and diseases^[47].

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COMMENTS

Background

Patients with long-standing inflammatory bowel disease (IBD) have a 2 to 8-fold increased relative risk of developing colorectal cancer as compared to the general population. Although prophylactic intervention with 5-aminosalicylate (5-ASA) is considered to be a promising chemopreventive strategy, additional studies are needed to elucidate its utility in IBD-promoted colorectal cancer. Plecanatide is a synthetic analog of the endogenous peptide uroguanylin (UG) and, like UG, is an activator of receptor guanylate cyclase-C (GC-C) signaling cascade that regulates fluid/ion secretion and epithelial cell homeostasis in the gastrointestinal (GI) tract. Oral treatment with plecanatide ameliorates GI inflammation in animal models of experimental colitis. Conceptually, chronic prophylactic intervention with an orally safe and locally-acting agent that not only suppresses inflammation but also regulates renewal of the GI mucosa is desirable.

Research frontiers

Therapeutic intervention with locally acting, minimally absorbed analogs of UG, represents a novel and safe approach for delaying the transition from IBD to

colon carcinogenesis.

Innovations and breakthroughs

This is the first report highlighting the therapeutic potential of an orally administered and mucosally active GC-C agonist for delaying the progression of ulcerative colitis to colorectal cancer in humans.

Applications

UG is an endogenous peptide hormone that regulates fluid/ion homeostasis and epithelial cell homeostasis and maintains the barrier function within the GI tract. Several studies have demonstrated that transcript levels of UG and its related peptide guanylin are markedly reduced in inflamed colonic tissues from patients with ulcerative colitis and Crohn's disease, as well as in human colonic polyps and tumors. It can be implied from these findings that the pathogenesis of these diseases might be associated with a deficiency of UG and GN. Oral therapy with plecanatide and other UG analogs could be considered as a replacement therapy to overcome the deficiency underlying the etiology of IBD and delay its progression to colorectal cancer.

Peer-review

This manuscript is well written and illustrated.

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Retrospective Study

Lymphocyte-to-monocyte ratio can predict mortality in pancreatic adenocarcinoma

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Abstract

AIM

To determine if the lymphocyte-to-monocyte ratio (LMR) could be helpful in predicting survival in patients with pancreatic adenocarcinoma.

METHODS

We retrospectively reviewed the medical records of all patients diagnosed with pancreatic adenocarcinoma in the VA North Texas Healthcare System from January 2005 to December 2010. The LMR was calculated from peripheral blood cell counts obtained at the time of diagnosis of pancreatic cancer by dividing the absolute lymphocyte count by the absolute monocyte count. A Univariable Cox regression analysis was performed using these data, and hazard ratios (HR) and 95%CI were calculated. The median LMR (2.05) was used to dichotomize patients into high-LMR and low-LMR groups and the log rank test was used to compare survival

between the two groups.

RESULTS

We identified 97 patients with pancreatic adenocarcinoma (all men, 66% white, 30% African-American). The mean age and weight at diagnosis were 66.0 ± 0.9 (SEM) years and 80.4 ± 1.7 kg respectively. Mean absolute lymphocyte and monocyte values were 1.50 ± 0.07 K/ μ L and 0.74 ± 0.03 K/ μ L respectively. Mean, median and range of LMR was 2.36, 2.05 and 0.4-12 respectively. In the univariable Cox regression analysis, we found that an increased LMR was a significant indicator of improved overall survival in patients with pancreatic adenocarcinoma (HR = 0.83; 95%CI: 0.70-0.98; $P = 0.027$). Kaplan-Meier analysis revealed an overall median survival of 128 d (95%CI: 80-162 d). The median survival of patients in the high-LMR (> 2.05) group was significantly greater than the low-LMR group (≤ 2.05) (194 d *vs* 93 d; $P = 0.03$), validating a significant survival advantage in patients with a high LMR.

CONCLUSION

The LMR at diagnosis is a significant predictor for survival and can provide useful prognostic information in the management of patients with pancreatic adenocarcinoma.

Key words: Prognosis; Lymphocyte-to-monocyte ratio; Pancreatic adenocarcinoma; Mortality; Biomarker

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Core tip: Pancreatic adenocarcinoma is an aggressive malignancy and many patients are presented with aggressive treatment options at diagnosis; often times they are unsure whether they should take a palliative route or a more aggressive approach to their care. Through a retrospective analysis of patients with pancreatic adenocarcinoma, we found that a higher lymphocyte-to-monocyte ratio is associated with improved survival. The lymphocyte-to-monocyte ratio was collected at diagnosis, and is readily available on routine blood work, making it a simple way to help predict and guide treatment in patients diagnosed with pancreatic adenocarcinoma.

Singh G, Nassri A, Kim D, Zhu H, Ramzan Z. Lymphocyte-to-monocyte ratio can predict mortality in pancreatic adenocarcinoma. *World J Gastrointest Pharmacol Ther* 2017; 8(1): 60-66 Available from: URL: <http://www.wjgnet.com/2150-5349/full/v8/i1/60.htm> DOI: <http://dx.doi.org/10.4292/wjgpt.v8.i1.60>

INTRODUCTION

The fourth leading cause of death related to cancer is attributed to pancreatic adenocarcinoma in the United States^[1], with an overall 5-year survival of 7.2% and

2.4% in patients with metastatic disease^[2]. The disease is generally silent in the early stages and is usually detected once patient develops symptoms from local or distant metastasis. Overall, half of the patients are found to have metastatic disease at diagnosis^[1]. Risk factors for pancreatic adenocarcinoma include male sex, elderly age, family history, African American race, obesity, diabetes, tobacco use, and chronic pancreatitis^[3,4]. The treatment of pancreatic cancer is dependent on stage of disease, and is divided into three categories: Resectable, locally advanced, and metastatic disease. Locally advanced disease can be treated with neo-adjuvant chemotherapy followed by surgical resection. The mainstay of treatment for metastatic disease is palliative chemotherapy^[5,6]. There have been numerous advances in oncologic therapeutics, however improvement of survival in patients with pancreatic cancer has been particularly slow^[1].

Currently, there is no effective way to predict treatment response and survival at diagnosis aside from stage of disease. For patients with resected cancer, predictors of survival include resection margins, tumor size, and response to chemo-radiation^[7,8]. Given the high morbidity and mortality associated with pancreatic cancer, any prognostic information available to risk stratify patients could be beneficial in planning treatment approaches and palliative discussions.

Inflammation and the body's cellular immune response have been shown to play an important role in the pathogenesis of malignancy and its progression from primary to metastatic disease^[9,10]. These concepts have led the absolute peripheral blood lymphocyte-to-monocyte ratio (LMR) to act as a surrogate biomarker of prognosis in different malignancies, with several studies showing an association between the LMR and survival in multiple myeloma, diffuse large B cell lymphoma, osteosarcoma, non-small cell lung cancer, and breast cancer^[11-15].

The primary aim of this study was to determine if the peripheral blood LMR at the time of diagnosis could be used as a prognostic biomarker in patients with pancreatic adenocarcinoma regardless of treatment modality.

MATERIALS AND METHODS

We identified a cohort of patients diagnosed with pancreatic cancer from the Dallas VA tumor registry at the Veteran's Affairs North Texas Health Care system (VANTHCS) between January 2005 and December 2010. All patients were treated based on the stage of the disease and accepted standard of care treatment protocols which included surgery, chemotherapy, radiation, and palliative stent placement.

We included patients that were only diagnosed with pancreatic adenocarcinoma; other pancreatic tumors such as lymphoma or metastases of other primaries were excluded from the analysis. The study protocol was approved by the VANTHCS Institutional Review

Board.

Data collection

Data collected included variables such as age, sex, race, weight, tobacco use, alcohol use, and medical co-morbidities. Specific variables for pancreatic cancer included age at diagnosis, largest diameter of tumor size seen on cross-sectional imaging and survival time in days (using a cutoff date of 10/11/2014 when the date of death was not available). Lab values including CA 19-9, CEA, white blood cell count, platelets, absolute lymphocyte count, lymphocyte percentage, absolute monocyte count, and monocyte percentage were all collected at or within one week of diagnosis.

LMR

The LMR was calculated by dividing the absolute lymphocyte count by the absolute monocyte count on the same blood draw that was obtained at the initial diagnosis of pancreatic cancer and prior to the initiation of any treatment.

Statistical analysis

Univariable Cox regression statistical analysis was performed to determine if LMR was a predictor of survival in patients with pancreatic adenocarcinoma; hazard ratios (HR) and 95%CI were calculated. A $P < 0.05$ was considered statistically significant. The median LMR was used to dichotomize patients into two groups: Patients with high-LMR and low-LMR. A Kaplan-Meier analysis with log rank test was used to compare survival between the two groups. The association between variables in the subgroups was evaluated by the χ^2 test for categorical variables, the t test for continuous variables, or the Fisher's Exact test.

These analyses were performed using SAS (version 9.2 software, The SAS Institute, Cary, NC) and R (version 2.15.1, the R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The overall baseline demographics, histopathologic characteristics, and stage are outlined in Table 1. There were 109 total patients in the Dallas VA Tumor registry that had any type of pancreatic cancer diagnosed between January 2005 and December 2010. Twelve patients with pancreatic neuroendocrine tumors were excluded. In the final analysis, a total of ninety seven patients with pancreatic adenocarcinoma were included (demographics were 66% white, 30% African-American; all were male subjects).

The stage at presentation was I (1%), II (24%), III (14%), and IV (61%). Patients had different presenting symptoms including weight loss (53%), jaundice (44%), poor appetite (21%) and abdominal pain (58%). Treatment included surgery (22%), neo-adjuvant therapy (3%) and palliative chemotherapy (37%) (Table 1). Kaplan-Meier survival analysis revealed an

Table 1 Baseline demographics pancreatic adenocarcinoma ($n = 97$) n (%)

Characteristic	Mean \pm SEM
Age	66 \pm 0.9
Weight at dx (lbs)	80.4 \pm 1.7
LMR	2.36 \pm 0.16
CA 19-9	17030.2 \pm 8861
CEA	960 \pm 657
Tumor size (cm)	4.13 \pm 0.2
WBC	9.1 \pm 0.5
Platelets	276.8 \pm 12.2
ALC K/ μ L	1.5 \pm 0.07
AMC K/ μ L	0.74 \pm 0.03
Race	
White	64 (66)
Black	29 (30)
Other	4 (4)
Location	
Head	64 (71)
Body	10 (11)
Tail	16 (18)
Stage	
I	1 (1)
II	23 (24)
III	14 (14)
IV	59 (61)
Treatment	
Surgery	22 (23)
Stent	38 (39)
Any chemo/rad	44 (45)
Palliative chemotherapy	36 (37)
Neoadjuvant chemotherapy	3 (3)
Adjuvant chemotherapy	10 (10)
Risk factors	
Alcohol	53 (55)
Tobacco	76 (78)

overall median survival for patients with pancreatic adenocarcinoma of 128 d (95%CI: 80-162 d).

Ninety-three of the 97 patients with pancreatic adenocarcinoma (96%) had absolute peripheral blood lymphocyte and monocyte values available at diagnosis to calculate the LMR. Mean absolute lymphocyte and mean absolute monocyte values were 1.50 ± 0.07 K/ μ L and 0.74 ± 0.03 K/ μ L respectively. Mean, median and range of LMR was 2.36, 2.05 and 0.4-12 respectively.

Univariable Cox regression analysis showed that an increased LMR was a significant indicator of improved overall survival in patients with pancreatic adenocarcinoma (HR = 0.83; 95%CI: 0.70-0.98; $P = 0.027$). Moreover, a high LMR in this group was significantly associated with a lower risk of early mortality, *i.e.*, survival < 6 mo (OR = 0.66; 95%CI: 0.46-0.95; $P = 0.025$). The median survival of patients in the high-LMR group (> 2.05) was significantly greater than the low-LMR group (≤ 2.05) (194 d vs 93 d; $P = 0.03$) (Figure 1).

To investigate the value of LMR in metastatic disease (stage IV), a uni-variable logistic regression analysis was performed in this group. There was no significant association between LMR and development of metastatic disease (OR = 0.91; $P = 0.476$). The area under the ROC curve was 0.609 (Figure 2), suggesting that LMR may be a poor marker for the prediction of

Table 2 Clinical variables in patients with high and low lymphocyte-to-monocyte ratio

	LMR \leq 2.05 (n = 50)	LMR > 2.05 (n = 43)	P value
Chemoradiation	20	21	0.4
Surgery	7	14	0.05
Stent	19	16	1.0
Stage			0.2
Stage 1	0	1	
Stage 2	8	14	
Stage 3	8	5	
Stage 4	34	23	
Location			0.4
Head	35	27	
Body	3	6	
Tail	9	6	
Race			0.05
White	36	25	
Black	11	18	
Other	3	0	
CEA (\pm SEM)	1075 \pm 1041	884 \pm 868	0.9
CA 19-9 (\pm SEM)	24957 \pm 17470	10162 \pm 3448	0.4
Age (\pm SEM)	66.6 \pm 1.2	65.3 \pm 1.4	0.5
Weight (\pm SEM)	79.3 \pm 2.2	82.2 \pm 2.5	0.4
Alcohol	27	24	1.0
Tobacco	38	34	0.8

LMR: Lymphocyte-to-monocyte ratio.

metastatic disease.

A uni-variable analysis of demographic and clinical variables between the high-LMR and low-LMR was performed to further characterize factors that could affect survival between the two groups. There was a marginally significant difference in the percentage receiving surgery in the high-LMR groups vs low-LMR group ($P = 0.05$) as well as in race between both groups ($P = 0.05$). There was no statistical significant difference between patients receiving chemo-radiation ($P = 0.4$) or stenting ($P = 1$). Furthermore, there was no difference in demographic variables such as age ($P = 0.5$), weight ($P = 0.4$), or risk factors such as tobacco ($P = 0.8$) or alcohol ($P = 1.0$) usage between the two groups. Analysis of clinical variables such as stage at presentation, location of tumor, mean CEA levels, and CA 19-9 levels between both groups did not reveal any significant difference (Table 2).

DISCUSSION

In this study we show that a higher LMR obtained from a peripheral blood count at the time of diagnosis is a predictor of improved survival in patients with pancreatic adenocarcinoma.

The LMR represents the balance between anti-tumorigenic lymphocytes and pro-tumorigenic monocytes, and may reflect the body's immune response to cancer and host-specific cancer aggressiveness. The T-lymphocytes of the native immune system play a vital role in suppressing anti-tumor immune responses and inducing apoptosis in tumor cells; low levels of

T-lymphocytes have been implicated in a poor immune response to cancers^[9,16,17]. Monocytes have been implicated in tumorigenesis, including differentiation into tumor-associated macrophages that support tumor invasion, angiogenesis and suppression of the body's own immune response against the tumor cells^[10,18,19]. Various studies have shown that the lymphocytes have an anti-inflammatory function and their role in impeding progression of tumor may be vital in the immune surveillance of different types of malignancies. Lymphocytes have different roles in identifying and eliminating tumor cells. This phenomenon is sometimes referred to as "immunoediting", and includes a complex interplay of various cells such as the NK cells, the NKT cells, macrophages, CD4 T cells and CD8 T cells. Several studies have shown that high numbers of CD8 T cells within the tumor portend a better prognosis^[20]. Further testing of these cells in a study including patients with pancreatic adenocarcinoma revealed FOXP3+ protein on immunohistochemical staining^[21]. This was further evaluated in a study, which looked at the lymphocyte density and the correlation with lymph node metastasis. They found out that the presence of FOXP3+ lymphocyte was higher in patients who had a higher histological grade of tumor, lymph node metastasis, and advanced stage tumors (stage III and IV vs stage I and II)^[22]. While the studies were not prospective in nature, they do validate the importance of these inflammatory cells in dictating the prognosis of these cancers.

Moreover, cytokines released by lymphocytes have roles in both promoting and suppressing a cancer. Haabeth *et al*^[23] conducted a study measuring the cytokine response in mice against cancers (myeloma and B-cell lymphoma). They found that inflammation driven by tumor specific Th1, allowed release of IFN-gamma which stimulated macrophages that were cytotoxic to the cancer cells. The CD4⁺ Th1 cells also help cytotoxic T cells in tumor rejection. On the other hand, the CD4⁺ Th2 cells are implicated in production of cytokines leading to B-cell activation. Similarly, Ling *et al.* showed that high numbers of Th1 lymphocytes in tumor tissue was associated with improved prognosis in patients with colorectal cancer^[24].

The prognostic ability of the LMR has been demonstrated in various malignancies^[11-15]. However, the exact utility of the LMR for primary pancreatic adenocarcinoma is unclear given the limited data available to date. Li *et al*^[25] evaluated the prognostic utility of the LMR in patients with pancreatic adenocarcinoma in the People's Republic of China but only included patients who underwent pancreatic resection and excluded patients who received adjuvant treatment, had significant co-morbid conditions or a life expectancy of < 6 mo. In addition, the preoperative LMR was used and not the LMR at diagnosis. They found that an elevated preoperative LMR was associated with longer survival. Fujiwara *et al*^[26] evaluated the postoperative LMR exclusively in patients who received pancreatic

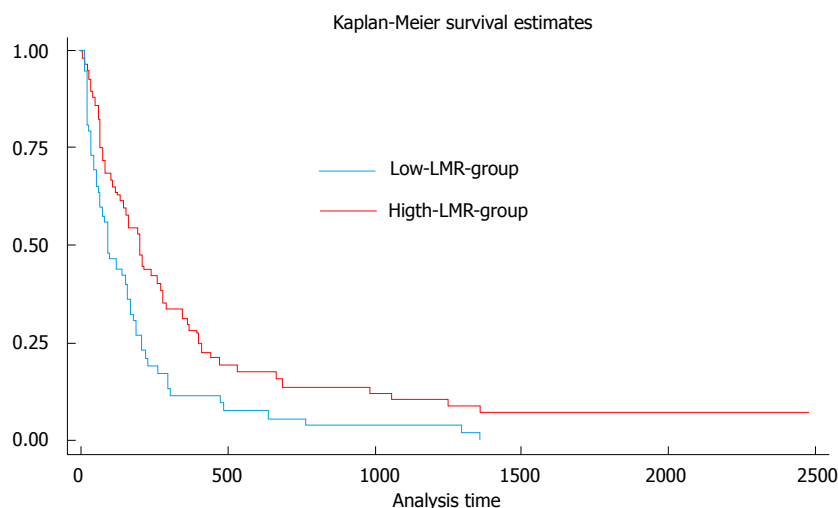


Figure 1 Kaplan meier survival curves of patients with low and high lymphocyte-to-monocyte ratio.

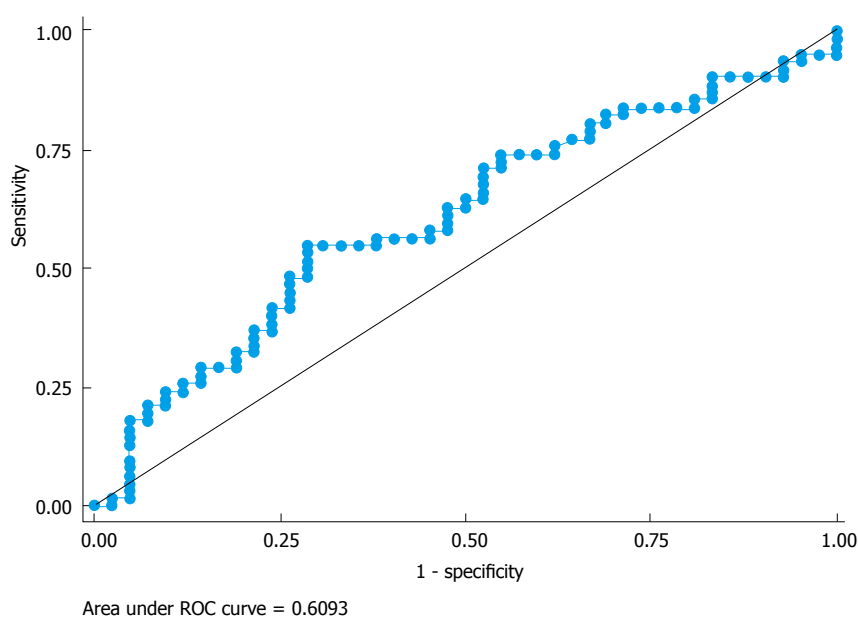


Figure 2 Price rate of change curve of the accuracy of lymphocyte-to-monocyte ratio in prediction of metastatic disease.

resections in Japan. They also reported an association between higher LMR and disease free survival.

In our study we included all adult patients diagnosed with pancreatic adenocarcinoma regardless of co-morbidities, life expectancy, functional status or intervention modality. Our study is the first study evaluating the utility of the LMR in an American cohort and shows that a higher LMR obtained at diagnosis of pancreatic cancer, regardless of the patient's functional status, various clinical factors or patient demographics validates a significant survival advantage. This information may be used in conjunction with other clinical factors to help in discussing prognosis and/or palliative options with patients as well as aid in scenarios where patients and providers decide on surgery vs neoadjuvant chemotherapy for borderline resectable tumors.

There are several limitations of this study. These include problems inherent to a retrospective study design such as treatment bias, a limited number of patients, as well as a unique patient population comprising exclusively of veteran male patients. The strengths of the study include a comprehensive multidisciplinary

evaluation of all patients in a tertiary care center with follow-up data available for each patient included in the analysis.

There is a role of immune-targeted therapies in the future, as it is clear that specific inflammatory cells have an impact on immune surveillance of tumors. Immunotherapies including chemicals that resemble cytokines can be used to up-regulate the cancer fighting cells. Clarifying the specific type of immune cells and chemical cytokines that attract tumor suppressing cells requires further research and understanding of the tumor biology, specifically for the different types of malignancies. Pancreatic adenocarcinoma, despite being one of the more aggressive cancers, still is in the nascent stages of research and investigators are continuing to learn the tumor biology and immunologic effects.

In the future, a large multicenter prospective trial would be beneficial to confirm our findings and validate it for routine use in the prognostication of patients with pancreatic cancer. The cutoff level of the LMR has varied in different studies, with each study using

a level specific to their cohort. In the future a single cutoff value for the LMR would need to be validated for further research purposes and clinical use. Moreover, the significance of LMR within various disease stages or a specific treatment modality (such as surgery, chemotherapy and radiation) could be further explored in adequately powered research studies.

In conclusion, the LMR is an easily acquired, minimally invasive, and inexpensive biomarker that may reflect the body's immune response to cancer and host-specific cancer aggressiveness. Our study shows that a high LMR predicts better overall survival for patients with pancreatic adenocarcinoma and can be used by clinicians and patients as a marker for prognosis.

COMMENTS

Background

Pancreatic adenocarcinoma is an aggressive malignancy and many patients are presented with aggressive treatment options at diagnosis. Inflammation plays an important role in cancer progression and metastasis, and the authors hypothesized that the lymphocyte to monocyte ratio may be a potential surrogate marker of prognosis, helping the patients make difficult decisions regarding which treatment option to pursue. Lymphocytes can be cytotoxic to tumor cells and can induce apoptosis in them, whereas monocytes have properties that promote tumorigenesis. These features might explain why a high lymphocyte-to-monocyte ratio (LMR) in peripheral blood has been found to be a favorable prognostic marker for a number of malignancies.

Research frontiers

Several malignancies have shown to have a favorable prognosis with a high peripheral blood LMR. Further research in determining the actual cellular mechanisms regarding the cytotoxic and apoptotic effects of inflammatory cells in different malignancies is where the basic science aspect would be beneficial. Also, validating a certain cutoff point for the LMR will allow clinicians to use the LMR in practice as a prognostic marker.

Innovations and breakthroughs

The LMR has been shown to be of prognostic significance in different malignancies such as breast cancer, multiple myeloma, lymphomas, osteosarcomas, and lung cancers. There are also other inflammatory markers in investigation such as the neutrophil to lymphocyte ratio, which has also been shown to have some prognostic significance.

Applications

They can use the LMR as a surrogate marker of prognosis in patients with pancreatic adenocarcinoma. It is available on routine blood work as they can calculate the LMR from a peripheral blood draw. The value of the LMR, if high, suggests a better prognosis, and may help guide treatment decisions.

Terminology

LMR is the absolute lymphocyte count divided by the absolute monocyte count.

Peer-review

The paper is well-written.

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Observational Study

Current practice and clinicians' perception of medication non-adherence in patients with inflammatory bowel disease: A survey of 98 clinicians

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Author contributions: Soobraty A and Boughdady S collected the data, performed the analysis and wrote the draft manuscript; Selinger CP designed the study, supervised data collection and analysis and critically reviewed the manuscript.

Institutional review board statement: The study was exempt from the requirement of research ethics committee approval as no patient data were elicited.

Informed consent statement: All study participants provided informed consent by return of the online questionnaire.

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Abstract

AIM

The survey ascertains perceptions and describes current practice of clinicians regarding medication non-adherence in patients with Inflammatory Bowel Disease.

METHODS

Gastroenterologists, trainees and inflammatory bowel disease (IBD) specialist nurses from the United Kingdom were invited to a web based survey collecting data on clinician demographics, patient volume and level of interest in IBD. Respondents were asked to estimate non-adherence levels and report use of screening tools and interventions to improve adherence.

RESULTS

Non-adherence was seen as an infrequent problem by 57% of 98 respondents. Levels of non-adherence were estimated lower than evidence suggests by 29% for mesalazine (5ASA), 26% for immunomodulators (IMM) and 21% for biologics (BIOL). Respondents reporting non-adherence as a frequent problem were more likely to report adherence levels in line with evidence (5ASA $P < 0.001$; IMM $P = 0.012$; BIOL $P = 0.015$). While 80% regarded screening as important only 25% screen

regularly (40% of these with validated assessment tools). Respondents stated forgetfulness, beliefs about necessity of medication and not immediately apparent benefits as the main reasons for non-adherence. Patient counselling on benefits and risks of medication was a commonly used intervention.

CONCLUSION

Clinicians treating IBD patients frequently underestimate non-adherence and use of validated screening tools is infrequent. Most respondents identified the main factors associated with non-adherence in line with evidence and often counselled patients accordingly. Professional education should focus more on non-adherence practice to avoid adverse treatment outcomes associated with non-adherence.

Key words: Non-adherence; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Clinical practice

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Core tip: Non-adherence to maintenance medication is a very common phenomenon occurring in up to 50% of patients with inflammatory bowel disease. This survey demonstrates that many clinicians underestimate the extent of non-adherence and screening for non-adherence is infrequent and not systematic. The lack of evidence for any intervention to improve adherence is reflected by the participants divergent practice to improve adherence. There is an urgent need for further clinician education on non-adherence and robustly tested interventions that are capable of improving adherence.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to a group of conditions that mainly affect the gastrointestinal tract. The two primary conditions Crohn's disease and ulcerative colitis are chronic and debilitating diseases that can have very serious consequences including hospitalisation, surgery and an increased risk of developing colorectal cancer (CRC)^[1]. However, evidence has shown that if IBD is treated appropriately and patients are adherent to IBD maintenance medications, disease morbidity is improved by reducing the frequency and severity of relapses^[1,2]. Adherence can lessen the risk of developing CRC and can improve other treatment outcomes for patients, for example, by providing a

better quality of life^[3,4].

However, despite this, there is evidence that approximately 30%-40% of patients do not adhere to their prescribed medication^[4-11]. The literature also suggests that the level of non-adherence varies according to the type of medication. IBD can be treated using a wide range of medications, including mesalazine (5ASA), immunomodulators (IMMs) and biological agents (BIOL) such as infliximab, and adalimumab. Non-adherence occurs in 30%-45% with mesalazine^[8-10], 15%-20% with IMMs^[10,12], 5%-10% with biologics^[13].

Low adherence to medication leads to poor disease control, which not only has an impact on the patient, but also the economy incurred through the cost of absenteeism, medical care and hospitalisation. Non-adherence is therefore a cause of financial burden to health services^[14-16].

While forgetfulness is the main reason for non-intentional non-adherence, several reasons for intentional non-adherence have been identified^[4]. Apart from psychological comorbidities and quiescent disease activity the most constant findings relate to the patient perception of their medication. Non-adherent patients were more likely to express doubts over the necessity for maintenance medication and had greater concerns over potential adverse effects^[5,10].

Though there are numerous methods to determine adherence, studies have found that clinicians find this difficult to gauge amongst their patients^[17,18]. Methods used to screen for adherence as identified by the literature include measuring drug metabolic levels, using scales such as the Morisky Scale^[18] or Medication Adherence Report Scale (MARS)^[5] and the use of simple questioning. Currently, little is known about screening behaviour of health professionals in the United Kingdom or how they manage non-adherence. In addition, there is not much information on how clinicians perceive the problem of non-adherence amongst those they are treating for IBD^[17,18].

The aim of this study was to assess clinicians' awareness of the extent of non-adherence in IBD. We also aimed to explore clinicians' perception of factors associated with non-adherence and identify potential differences in perception by profession, experience or level of interest in IBD. Finally, we aimed to investigate the use of screening tools and the management of non-adherence in patients with IBD amongst health professionals.

MATERIALS AND METHODS

We developed an online survey assessing clinicians' perceptions and practice based on a literature search. The survey was piloted with 8 IBD specialists and some clarifying minor amendments were based on their feedback. The survey containing both open and closed questions is available as a supplement (S1).

The survey questionnaire collected data on the parti-

Table 1 Respondents' demographics, self-reported expertise and scope of inflammatory bowel disease practice

		Frequency	Percentage
Sex	Male	46	47%
	Female	52	53%
Age, yr	20-29	7	7%
	30-44	45	46%
	45-60	40	41%
	> 60	6	6%
Years in practice, yr	< 5	11	11%
	5-9	21	22%
	10-14	17	17%
	15-19	10	10%
	≥ 20	39	40%
Profession	Gastroenterology trainee	17	17%
	Gastroenterology consultant	51	52%
	IBD nurse specialist	28	29%
	Other	2	2%
Geographic region	England	92	95%
	Scotland	2	2%
	Wales	2	2%
	Northern Ireland	1	1%
Self-rated level of IBD interest in medically qualified staff	General gastroenterologist	32	46%
	Interest in IBD	18	27%
	Expert IBD physician	18	27%
IBD patients per week	Range 0-150		
	Mean 25		

IBD: Inflammatory bowel disease.

Participants' demographics, level of interest in IBD and the number of patients with IBD typically seen in a week. Participants were asked about their overall impression of non-adherence amongst their local patient population. Furthermore we asked respondents to estimate the levels of non-adherence amongst those being managed with mesalazine (5ASA), IMMs and biologics (BIOL) therapy. Perceived reasons for non-adherence were explored by asking respondents to rank the significance of 8 pre-specified reasons (derived by the authors from the literature) with 1 being the most important and 8 being the least. Analysis of ranking preferences was performed by counting the number of times respondents had stated a particular reason to be in the top 3. Moreover, the survey gathered data on participants' practice regarding the use of screening tools and any interventions used in their practice to improve adherence.

The survey was distributed by email to consultant gastroenterologists, trainees *via* the British Society Gastroenterology IBD section (775 members) and IBD specialist nurses from the United Kingdom *via* the UK IBD nurse network (approximately 200 members). We aimed to include different staff groups (consultants, trainees and nurses) in order to collect the views and opinions of those involved in all aspects of patient care. The survey and data compilation were performed through Bristol Online Surveys, an academic online survey system, over a 3 mo period.

Quantitative data were analysed with SPSS Statistics (IBM, version 22) using χ^2 tests to compare responses between the different participant groups. The qualitative data responses were collated and key themes were identified and described.

In the United Kingdom Ethical approval is not required for survey studies examining the views and opinions of clinicians only.

RESULTS

Respondents

Of the 98 study participants (response rate 10%) 52 (53%) were female, 46 (47%) male, and 47% of participants were older than 44 years. Respondents included 51 consultants, 17 trainees, 28 IBD specialist nurses, 1 IBD dietitian and 1 biologics nurse specialist (Table 1). Approximately half of respondents had 15 years' experience or more. Of the 68 medically qualified respondents 32 classed themselves as general gastroenterologists, 18 had an IBD interest and 18 stated that they were IBD experts. The number of patients seen in an average week varied greatly amongst the study's participants between less 10 to over 100 patients with a mean of 25 patients per week.

Respondents' perception of non-adherence

Non-adherence in their local patient cohort was perceived as a frequent problem by 43%, as an infrequent problem by 49%, while 7% reported few cases only and 1% reported no adherence issues. Overall 57% of respondents reported non-adherence at best to be an infrequent problem. Older respondents were more likely to report non-adherence as an at best infrequent problem ($P = 0.043$). No other correlations between overall perception of non-adherence and other factors displayed in Table 1 were found.

Respondents estimated level of non-adherence considerably lower than suggested by the evidence base in 31% for 5ASA, in 28% for IMM and in 23% for BIOL (Table 2). Respondents who perceived non-adherence as a frequent problem were more likely to report adherence levels in line with the evidence base (5ASA $P < 0.0001$, IMM $P = 0.002$, BIOL $P = 0.006$; Table 3). Self-declared level of interest in IBD did not affect whether or not participants estimated non-adherence for 5ASA and IMM in line with evidence. However, a higher level of interest in IBD was found to significantly correlate with estimating level of non-adherence for biologics therapy in line with evidence ($P = 0.012$; Table 4). No other correlations between perception of non-adherence for 5ASA, IMM or BIOL and other factors displayed in Table 1 were found.

Perceived reasons for non-adherence

The most commonly identified reasons for non-adherence were patient's forgetfulness (rank 1), lack of belief in the necessity for medication (rank 2), benefits of

Table 2 Estimation of non-adherence levels by respondents and percentage in line with evidence

Medication	Column A: literature-based non-adherence levels	Column B: perceived mean non-adherence levels	Proportion who estimated non-adherence levels below the levels in column A
Mesalazine	30%-45%	20%	31%
Immunomodulator therapy	15%-20%	10%	28%
Biological agents	5%-10%	1%	23%

Table 3 Association between perception of non-adherence as a frequent problem and reporting non-adherence levels in line with the evidence base

Medication	χ^2 value	Degrees of freedom	P value
Mesalazine	33.226	1	0.000
Immunomodulator therapy	12.592	2	0.002
Biological agents	7.459	1	0.006

medication not immediately apparent (rank 3) and concerns over potential side effects (rank 4; Table 5).

Screening practice

Nearly all (99%) respondents thought that improving adherence to medication would improve health-related outcomes in IBD and 80% regarded screening as important. However, only 58% reported ever using screening tools and only 25% stated that they screen their patients on a regular basis. In addition, it was found that only 40% use validated assessment tools to screen for adherence on a regular basis. Among the respondents who used screening tools, 60% used simple questioning asking their patients whether they were taking all their medications and 37% used Drug metabolic levels to assess non-adherence. No participants reported using the Morisky scale or the MARS and only 3% used the Visual Analogue Scale.

Thematic coding of qualitative data found that while some screen for non-adherence routinely, others only screen if a patient is not responding to medication or if they are treating someone who has regular flares or relapses. Reasons stated for not using screening tools included not having enough time during consultations and a lack of knowledge on the different screening tools available.

Managing non-adherence

Ninety-six percent of respondents believed that non-adherence can be addressed and that determining low adherence is important because interventions can increase adherence. Participants were asked to rank the effectiveness of certain interventions as a part of our survey. Fifty-two percent thought that involving patients in their treatment was the most effective intervention (rank 1). Other highly ranked interventions included "general education on the disease" (rank 2) and "less frequent dosing" (rank 3) and patient counselling was ranked 4th and most commonly included information on

Table 4 Association between level of interest in inflammatory bowel disease and estimation of non-adherence to biological therapy

Pearson χ^2 test	What percentage of your patients on biological therapy are non-adherent?	
Level of interest in IBD for medical staff	χ^2 value	8.863
	Degrees of freedom	2.000
	P value	0.012

IBD: Inflammatory bowel disease.

benefits and risks of medication.

DISCUSSION

Non-adherence is a common issue amongst cohorts of patients with chronic diseases and especially in those with IBD. In contrast to the well-established evidence on the extent of adherence^[4,19,20] and factors associated with it^[10], little is known on how clinicians perceive non-adherence and how they combat it^[17]. Yet identification of non-adherence is the vital first step in attempting to avoid the increased health burden for the patient and financial burden for the healthcare system associated with non-adherence. This study is only the 2nd survey of clinicians views overall and the first in the United Kingdom.

Our study shows that clinicians have a tendency to underestimate the extent of non-adherence as only 43% of respondents thought that non-adherence was a frequent issue. It is interesting to note that older health professionals are more likely to underestimate the problem of non-adherence more often than other clinicians. No other respondents' characteristics (nurse vs doctor, scope of IBD practice, self-reported level of IBD expertise) were associated with the overall impression of non-adherence. Perception of non-adherence may therefore be a generational issue that could be influenced by different methods of training over time and associated changes in practitioner-patient relationships. Further work is required to elicit why non-adherence rates are wrongly perceived as low in general. Clinicians may feel uncomfortable with the thought of patients not following agreed treatment plans and may also feel helpless when tasked with improving non-adherence given the lack of evidence based interventions.

Table 5 Perception of reasons for non-adherence

Rank	Actual side effects of medication	Patient forgets to take medication	Benefits of medication not immediately apparent	Anxiety or depression	Poor patient knowledge of disease	Frequency of dosing	Beliefs about necessity of medication	Concerns over potential side effects
Ranked 1 st	14.60%	32.30%	13.70%	5.30%	7.30%	9.60%	12.40%	11.30%
Ranked 2 nd	9.40%	25%	20%	7.40%	11.50%	18.10%	23.70%	14.40%
Ranked 3 rd	7.30%	11.50%	18.90%	8.40%	8.30%	21.30%	20.60%	23.70%
Ranked 4 th	14.60%	7.30%	11.60%	7.40%	18.80%	11.70%	15.50%	12.40%
Ranked 5 th	10.40%	12.50%	16.80%	13.70%	14.60%	9.60%	10.30%	16.50%
Ranked 6 th	24%	3.10%	9.50%	16.80%	18.80%	13.80%	8.20%	9.30%
Ranked 7 th	10.40%	4.20%	5.30%	16.80%	14.60%	8.50%	3.10%	9.30%
Ranked 8 th	9.40%	4.20%	4.20%	24.20%	6.30%	7.40%	6.20%	3.10%
Ranked in top 3	30	66	50	20	26	46	55	48
by <i>n</i> =								
Overall rank	6	1	3	8	7	5	2	4

Levels of non-adherence were underestimated for all medications enquired about in our survey (5ASA, IMM and BIOL). The authors elicited observed non-adherence rates found in the majority of published cohort studies^[8-10,12,13] and compared the survey respondents' perceptions with these levels. Between 23%-31% estimated the levels of non-adherence to the different medication in their local patient population much lower than the evidence suggests. This may go some way in explaining why non-adherence to maintenance medication often goes undetected. We have demonstrated that those practitioners who perceive non-adherence as a frequent issue, however, estimate non-adherence levels closer to the levels derived from the evidence base.

Respondents ranked unintentional non-adherence (patient's forgetfulness) and three reasons associated with intentional nonadherence (lack of belief of necessity for medication, benefits not immediately apparent, concerns over potential side effects) as the most common reasons for non-adherence. This closely mirrors the evidence base as these factors are most consistently associated with non-adherence^[4,10,20]. Respondents ranked factors that are only inconsistently or not associated with non-adherence (frequency of dosing, anxiety or depression, patient knowledge) as less important thereby demonstrating a good understanding of the factors associated with non-adherence.

Though the majority of respondents (80%) stated that they thought screening was an important issue and that adherence to medication would improve disease outcomes, this was not reflected in the participants' clinical practice. 76% said that they screened at least occasionally for non-adherence, but of those 52% said that they only use it "rarely" or "sometimes". A similar study to this one, carried out by Trindade *et al.*^[18] in the United States found that 77% of participants self-reportedly screened for adherence, however, the frequency of screening is unknown^[18]. The commonest screening method used was simple questioning of the patient (as in Trindade's study), which is known to be unreliable in assessing adherence as it vastly

underestimates non-adherence^[18,21,22]. Evidence based adherence report tools were only used by a minority and these were largely restricted to blood tests. A strong effort should be made to encourage health professionals to use validated screening tools such as the 8-item Morisky Medication Adherence Scale (MMAS-8) and MARS, which are effective at detecting non-adherence non-invasively^[18].

The conundrum presented by our findings is that while 96% of respondents believed that non-adherence can be addressed and that interventions can improve adherence, only 25% of respondents reported that they screen their patients regularly. It is perceivable that respondents believe that non-adherence can be improved yet have limited experience, resources or faith in interventions' success to actually implement regular screening for non-adherence. In view of the low screening rates, interventions targeting clinicians' knowledge, skills and practices need to be found. This should include education about non-adherence, efforts at raising general awareness, especially associated consequences in terms of morbidity, and financial cost. Clinicians should also be trained in the use of validated screening tools available such as the MMAS-8 and MARS.

The field of interventions aimed at improving non-adherence is difficult as the evidence consists of under-powered studies^[23], studies with non-reproducible complex interventions^[24], ongoing studies^[25] and review articles having to base advice on associated factors alone due to the lack of rigorously tested interventions. This dilemma is revealed by the high ranking of interventions without any positive evidence base ("general disease education", "less frequent dosing") and the high ranking of important but insufficiently defined interventions such as "patient involvement in treatment" and "counselling". Those engaged in patient counselling reassuringly report using themes around medication information concerning the evidence based necessity and concerns framework^[5,10,26]. A number of technological advances allow for frequent reminders for patients, but most of these systems fail to address intentional non-adherence.

Whether patient counselling can be effectively delivered in a remote, technology based way has not been rigorously tested so far. Arguably, personal contact with clinicians and especially IBD nurses may facilitate counselling more effectively.

There are a number of limitations to our study. First of all the response rate of 10% is low, but this is in line with other surveys of health care professionals^[27,28]. A degree of selection bias is inherent in survey studies but the spread of self-reported expertise among respondents in our study suggest a reasonably balanced sample. We believe that the sample is likely representative of IBD clinicians in the United Kingdom, but there are no reliable data to verify this assumption. Whether non-responders hold the same views as responders is unclear. Furthermore, subjective bias may have occurred as respondents may have given answers that they thought were expected of them or answers that they think the researchers were looking for, which in turn may explain the discrepancy between the generally positive perceptions of screening and the lack of regular screening in practice. We asked respondents to rank pre-specified reasons for non-adherence and pre-specified interventions based on our valuation of the existing literature to allow for a meaningful analysis. Naturally this list will have not been comprehensive and items such as "clinician-patient relationship" were not included.

In conclusion, we found that clinicians often underestimate the problem of non-adherence in patients with IBD. We also found that the use of validated screening tools was infrequent. This is a phenomenon, which occurs across all grades and professions. In addition, we found that the factors associated with non-adherence were correctly identified by participants. Based on our findings, it seems sensible to focus educational efforts for clinicians on the issue of non-adherence and its negative impact on patients with IBD. Further research is needed to establish simple and effective interventions to manage non-adherence.

COMMENTS

Background

Non-adherence to inflammatory bowel disease (IBD) maintenance medication occurs in up to 50% of patients. It is associated with adverse clinical outcomes and increased healthcare costs. While there are a number of methods that can detect non-adherence clinicians often struggle in routine clinical practice to detect it. There is a lack of robustly tested interventions capable of improving non-adherence to IBD medication.

Research frontiers

In the absence of clear guidelines and evidence for interventions little is known how clinicians perceive and how they address the issue of non-adherence. This survey ascertained perceptions and describes current practice to inform education, research and guidelines for clinical practice.

Innovations and breakthrough

A multitude of studies have aimed to identify factors associated with non-adherence. The most frequently found modifiable factors for intentional non-

adherence are a lack of belief in the necessity for medication and concerns over potential side effects. Patient friendly and easily implementable self-report tools to detect non-adherence have been assessed and validated ready for use in routine clinical practice.

Application

Further education about non-adherence is required as clinicians treating IBD patients frequently underestimate non-adherence. The use of validated screening tools should be encouraged. The respondents clearly identified the main factors associated with non-adherence and aimed to address them by counselling. A formally tested evidence based intervention to improve non-adherence is urgently required.

Terminology

IBD comprises ulcerative colitis and Crohns's disease, which are chronic inflammatory disorders of the gastrointestinal tract. Non-adherence is defined as a patient driven deviation from an agreed treatment plan.

Peer-review

This manuscript is well written and gives a clear overview of the perception of clinicians about medication non-adherence in IBD. As non-adherence is still a major problem in chronic diseases.

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Randomized Controlled Trial

Itopride for gastric volume, gastric emptying and drinking capacity in functional dyspepsia

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Data sharing statement: Technical appendix and dataset available from corresponding author at shahab.abid@aku.edu.

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Abstract

AIM

To study the effect of itopride on gastric accommodation, gastric emptying and drinking capacity in functional dyspepsia (FD).

METHODS

Randomized controlled trial was conducted to check the effect of itopride on gastric accommodation, gastric emptying, capacity of tolerating nutrient liquid and symptoms of FD. We recruited a total of 31 patients having FD on the basis of ROME III criteria. After randomization, itopride was received by 15 patients while 16 patients received placebo. Gastric accommodation was determined using Gastric Scintigraphy. ¹³C labeled octanoic breadth test was performed to assess gastric emptying. Capacity of tolerating nutrient liquid drink was checked using satiety drinking capacity test. The

intervention group comprised of 150 mg itopride. Patients in both arms were followed for 4 wk.

RESULTS

Mean age of the recruited participant 33 years (SD = 7.6) and most of the recruited individuals, *i.e.*, 21 (67.7%) were males. We found that there was no effect of itopride on gastric accommodation as measured at different in volumes in the itopride and control group with the empty stomach ($P = 0.14$), at 20 min ($P = 0.38$), 30 min ($P = 0.30$), 40 min ($P = 0.43$), 50 min ($P = 0.50$), 60 min ($P = 0.81$), 90 min ($P = 0.25$) and 120 min ($P = 0.67$). Gastric emptying done on a sub sample ($n = 11$) showed no significant difference ($P = 0.58$) between itopride and placebo group. There was no significant improvement in the capacity to tolerate liquid in the itopride group as compared to placebo ($P = 0.51$). Similarly there was no significant improvement of symptoms as assessed through a composite symptom score ($P = 0.74$). The change in QT interval in itopride group was not significantly different from placebo (0.10).

CONCLUSION

Our study found no effect of itopride on gastric accommodation, gastric emptying and maximum tolerated volume in patients with FD.

Key words: Itopride; Gastric emptying; Gastric accommodation; Functional dyspepsia; Dyspepsia

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Core tip: Through this study we wanted to find the effect of itopride on gastric accommodation, gastric emptying and drinking capacity in patients with functional dyspepsia (FD) in Pakistani population. The strength of our study was that we used objective measures, *i.e.*, gastric scintigraphy and ^{13}C labeled octanoic acid breath test to measure gastric accommodation and gastric emptying. Diagnosis of FD was based on ROME III criteria and was done after using extensive investigations to rule out organic cause for the symptoms. We found no effect of itopride on gastric accommodation, gastric emptying and maximum tolerated volume in patients with FD in our study.

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INTRODUCTION

Patients presenting with epigastric pain and burning, early satiation and postprandial fullness without any

structural, organic or systematic pathology are labeled as having functional dyspepsia (FD)^[1,2]. Globally prevalence of FD varies from 1.8% to 57% depending on the geographic location and diagnostic criteria used^[3]. There is no published data on community based prevalence of FD from South Asia but experts consider it to be an important problem for our population^[3,4]. FD reduces productivity and incurs a considerable cost on health system^[5]. Only in 2009 the cost incurred to the health system by this morbidity was \$18 billion^[6].

Muti-factorial pathogenesis of FD makes it a difficult condition to intervene^[7]. These patients have inability of the stomach to change its volume in response to food, decreased stomach compliance and inability of the stomach to empty^[7]. Delayed gastric emptying is associated with the symptoms of nausea, vomiting and postprandial fullness^[8]. These symptoms result in lower productivity of the patients and a compromised quality of life^[7,9]. Delayed gastric emptying is found in one third of the patients with FD^[8,10]. These patients showed slower gastric emptying as compare to the normal individuals^[11]. This is because of sub optimal gastric myoelectric activities^[12,13].

Different drug therapies used for FD that include eradication of *Helicobacter pylori* (*H. pylori*), use of proton pump inhibitors (PPIs) and anti-depressants failed to demonstrate a convincing effect^[14,15]. Guidelines recommend eradication of *H. pylori* but this treatment alone depends on the type of the FD being treated^[15]. Evidence in favor of efficacy for using PPIs for all the patients with FD is not clear^[16]. It's argued that PPIs may only be effective in patients having co-morbid reflux symptoms^[16]. Though data suggest that anti-depressants like mirtazapine might be beneficial for some sub-groups of FD but still more studies are needed to recommend its usage for all patients with FD^[17]. Symptoms of FD are improved by prokinetic agents^[18]. Metoclopramide can cause extra pyramidal movement disorders^[19]. Use of domperidone can result in rise in prolactin level leading to gynaecomastia^[20]. Cisapride can result in prolonged QT interval and arrhythmias^[21]. Itopride is a dopamine (D2) antagonist with peripheral action. It doesn't cause severe elevation of prolactin and or pathological changes on electrocardiogram (ECG)^[22]. A recent meta-analysis concluded that itopride improves the symptoms of early satiety and postprandial fullness^[23]. Through this study we wanted to find the effect of itopride on gastric accommodation, gastric emptying and capacity of tolerating nutrient liquid drink in patients with FD in Pakistani population.

MATERIALS AND METHODS

We conducted a randomized controlled trial to see the effect of itopride on gastric emptying. This study was conducted after approval from Aga Khan University Ethical Review Committee (Clinical trial registration number: NCT01226134). Subject for this study were

enrolled after written informed consent that was made on the basis of declaration of Helsinki.

Study population

Total of 31 patients were recruited for the purpose of this study from the gastroenterology clinics of Aga Khan University Hospital. Eligibility criteria used for recruiting these patients was; age equal to or greater than 18 years, diagnosed as FD on the base of Rome III criteria, negative for *H. pylori* on gastric biopsy and Urea Breath Test, negative duodenal biopsy for giardiasis or celiac disease or any other established organic pathology, and normal upper abdominal ultrasound. We excluded; pregnant women, patients taking other medications that alter gastric motility like macrolide and anti-emetics and antibiotics.

Randomization

Before undergoing randomization patients were assessed for symptoms that included epigastric discomfort, heart burn, acid regurgitation, upper abdominal pain, belching, nausea, early satiety, and postprandial fullness. Blood samples of these patients were taken to check for serum hemoglobin level, white blood cell count, platelet count, serum alanine aminotransferase (SGPT) and prolactin level. Electrocardiogram of the patient was performed to find out the QT interval at the baseline. Single photon emission tomography and satiety drinking test was performed to measure gastric accommodation at baseline. After completing the baseline investigations, out of all patients that were recruited 15 were randomly allocated to the intervention group while 16 were randomly allocated to the placebo group. Patients in the intervention group received 150 mg of itopride for 4 wk. Patients were instructed to take antacids as and when required.

Outcome measures

Gastric accommodation: Gastric accommodation was determined by estimating the change in gastric volumes using Gastric Scintigraphy and by the help of computer software which convert the gastric images into 3D images and calculate the estimated gastric volumes^[24]. Gastric volumes were determined before giving itopride or placebo agent and after completion of intervention period. We injected 99mTc pertechnetate followed by the use of Analyze software for reconstruction of tomographic images. These images were acquired after an overnight fast among all the participants and then after giving 300 mL of nutrient drink at an interval of 20, 30, 40, 50, 60, 90 and 120 min. Analyze PC 2.5 software system was used to find out stomach volume measurements^[24].

Gastric emptying: After an overnight fast, ¹³C labeled octanoic breath test was performed to assess gastric emptying^[25,26]. A test meal containing ¹³C was given to patient which is supposed to be completed in 10 min.

Breath sample was taken before test meal (150 mL of water with a sandwich of scrambled egg containing ¹³C octanoic acid and 250 mL of orange juice) and at an interval of every 15 min for 4 h and then half-hourly for another two hours. During the measurement time the subject remained sedentary while reading or watching television. If necessary limited movements between the breaths collections were permitted.

Satiety drinking capacity test: Subjects after an overnight fast were told to come at 8:30 AM in the morning. They were asked to grade their satiety from 0 to 5 (5 being the maximum satiety). A drink containing 6.5 g fat/100 mL, 1.1 g carbohydrate and 5 g of protein (nutridrink kcl 150/100 mL) which tasted of vanilla was taken by the participants at room temperature. Subjects drank at the rate of 15 mL/min. Symptoms were scored at every five minutes interval. Test was ceased once a score of 5 is achieved^[27].

Symptoms of FD: Dyspeptic symptoms which included epigastric pain, epigastric discomfort, heart burn, acid regurgitation, upper abdominal pain, belching, nausea, early satiety and postprandial fullness were assessed at baseline and at 4 wk with validated 7-point global overall symptom scale^[28].

Sample size calculation

Change in gastric volumes between baseline and postprandial (accommodation) was the primary endpoint for this study. To detect 16% difference in the Itopride and placebo with power of 80% and 5% level of significance a sample size of 15 subjects was needed in each group. This effect size of 16% [$100 \times (\text{difference in group means divided by overall mean of the two groups})$] corresponds to the difference in the two groups that was relevant clinically. The Analysis of coefficient of variance (ANCOVA) was done for this analysis.

Statistical analysis

For the purpose of this study, mean and standard deviation were reported for quantitative variables. Means and standard errors adjusted for covariates were reported using ANCOVA. The difference in the change in volume between itopride and the placebo group was compared using Man Whitney *U* test.

RESULTS

A total of thirty-one individuals were recruited for the purpose of this study. Mean age of these individuals was 33 years. Most of the recruited individuals, 21 (67.7%) were males. After randomization into Itopride and placebo groups, the groups were similar on variables like age, gender, serum haemoglobin, white blood cells, platelet count, serum creatinine, SGPT, prolactin level and QT interval on ECG (Table 1). There was no lost to follow up.

Table 1 Distribution of age and gender by intervention arm *n* = 31

Variable	Itopride	Placebo	<i>P</i> value
Age mean (SD)	34.2 (6.4)	31.9 (8.5)	0.40
Gender <i>n</i> (%)			
Male	10 (66.7)	11 (68.8)	1.00 ¹
Female	5 (33.3)	5 (31.3)	
Hb (g/dL) mean (SD)	13.6 (2.3)	14.1 (1.7)	0.50
WBC ($\times 10$ Eq/L) mean (SD)	8.1 (2.0)	7.7 (1.9)	0.52
Platelet count ($\times 10$ Eq/L) mean (SD)	257.3 (59.5)	250.5 (57.2)	0.75
Creatinine (mg/mL) median (IQR)	0.9 (0.5)	0.8 (0.3)	0.05
SGPT (IU/L) median (IQR)	20 (9.0)	25.5 (17.0)	0.09
Prolactin level (mg/mL) (IQR)	7.3 (3.6)	5.7 (2.7)	0.29
QT interval mean (SD)	394.1 (21.6)	399.2 (22.9)	0.53

¹Fischer Exact test. WBC: White blood cell; IQR: Interquartile range.

Table 2 Mean volumes to measure gastric accommodation by scintigraphy using ANCOVA adjusted for age and gender

Mean (\pm SE)	Itopride	Placebo
Change in volume (post-pre) using scintigraphy		
Fasting	-22.2 (\pm 15.4)	7.5 (\pm 14.9)
20 min	-201.7 (\pm 102.4)	-31.5 (\pm 99.1)
30 min	0.14 (\pm 22.8)	-36.4 (\pm 22.1)
40 min	-27.6 (\pm 32.2)	-37.4 (\pm 31.2)
50 min	-60.0 (\pm 31.2)	-3.0 (\pm 30.2)
60 min	-3.1 (\pm 36.5)	15.0 (\pm 35.4)
90 min	3.8 (\pm 29.8)	-31.8 (\pm 28.8)
120 min	16.7 (\pm 28.9)	23.3 (\pm 28.0)
Effect on gastric emptying on ¹³ C labeled octanoic acid breath test (post-pre)	0.4 (\pm 0.4)	0.2 (\pm 0.4)
Effect on drinking capacity (post-pre)	22.5 (\pm 18.1)	36.0 (17.5)
Change in Symptom score (post-pre)	-5.8 (1.0)	-4.7 (0.9)

Gastric accommodation

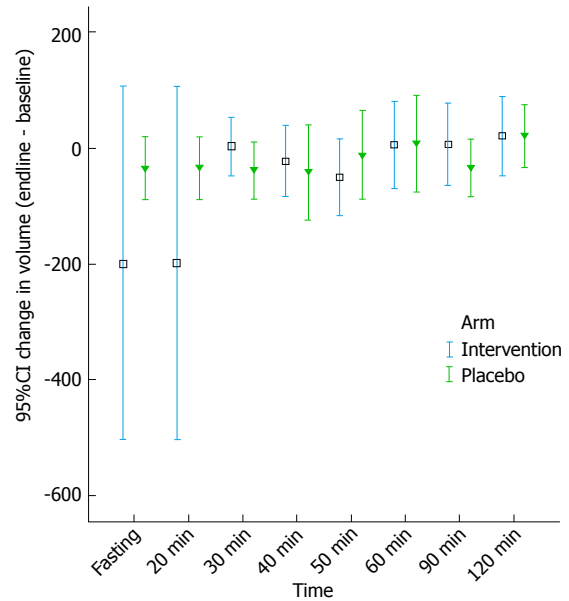
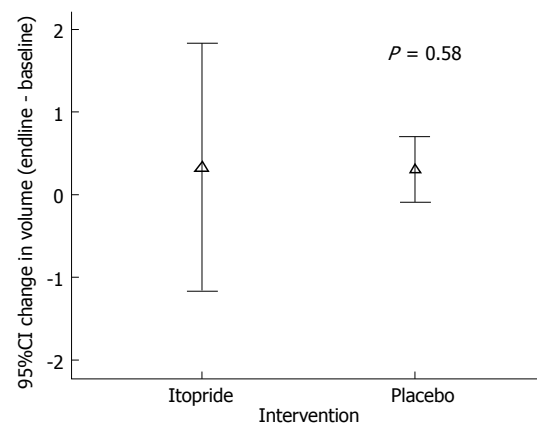
Gastric accommodation was checked using gastric scintigraphy by computing the change in gastric volume at empty stomach, 20, 30, 40, 50, 60, 90 and 120 min. We found that there was no statistically significant difference in the itopride and placebo group on gastric accommodation as measured with the difference in volume in the itopride and control group with the empty stomach ($P = 0.14$), at 20 min ($P = 0.38$), 30 min ($P = 0.30$), 40 min ($P = 0.43$), 50 min ($P = 0.50$), 60 min ($P = 0.81$), 90 min ($P = 0.25$) and 120 min ($P = 0.67$) (Figure 1). Mean volumes to measure gastric accommodation by scintigraphy using ANCOVA adjusted for age and gender were computed (Table 2).

Gastric emptying

Gastric emptying was done by doing breath tests on a sub sample ($n = 11$). There was no statistically significant difference ($P = 0.58$) between intervention and control group in gastric emptying (Figure 2).

Capacity of tolerating liquid drink

At the end of the intervention (Itopride or placebo)


Figure 1 Schintigraphy.

Figure 2 Breadth test.

there was no significant improvement in the capacity to tolerate liquid in the itopride group as compared to placebo ($P = 0.51$) (Figure 3).

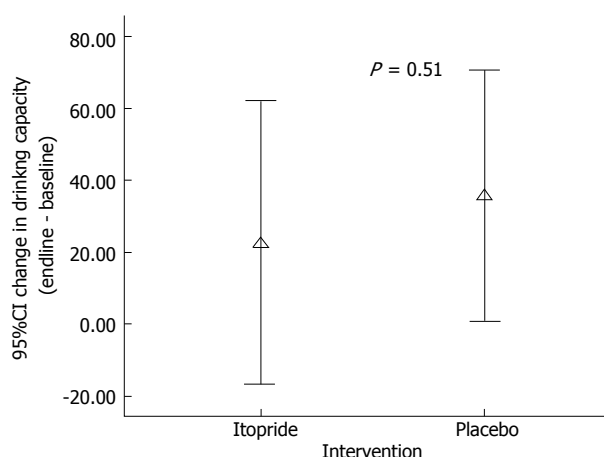
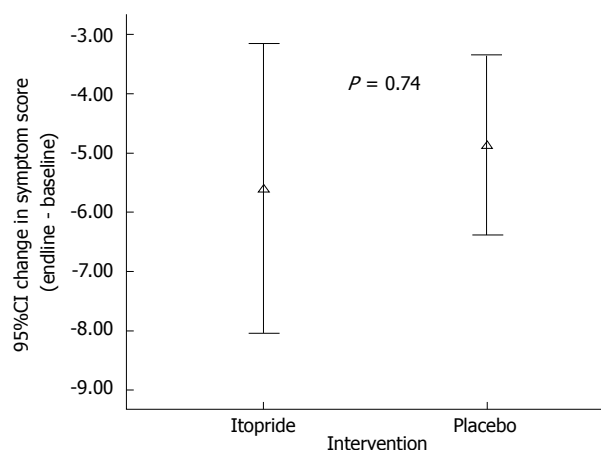
Symptoms of FD

There was no significant improvement of symptoms as assessed through a composite symptom score ($P = 0.74$) in the intervention group as compared to placebo (Figure 4). Similarly we didn't find any significant improvement in the individual symptoms that included epigastric pain ($P = 0.83$), epigastric discomfort ($P = 0.22$), heart burn ($P = 0.74$), upper abdominal pain ($P = 0.51$), nausea ($P = 0.08$), early satiety ($P = 0.34$) and postprandial fullness ($P = 0.25$) (Table 3).

The change in QT interval as a result of itopride group was not statistically different from placebo (0.10). Similarly itopride didn't alter the serum prolactin level in the intervention group as compare to the placebo group.

Table 3 Median Symptom score along with interquartile range at baseline and end of four weeks (placebo *vs* itopride) *n* = 31

Symptoms	Baseline			End of four weeks		
	Itopride	Placebo	<i>P</i> value	Itopride	Placebo	<i>P</i> value
Epigastric pain	3.0 (1.0)	4.0 (2.0)	0.18	2.0 (1.0)	2.0 (1.0)	0.83
Epigastric discomfort	2.0 (1.0)	1.0 (1.0)	0.03	1.0 (1.0)	1.0 (1.0)	0.22
Heart burn	1.0 (3.0)	1.0 (1.8)	0.37	1.0 (1.0)	1.0 (1.0)	0.74
Upper abdominal pain	3.0 (2.0)	1.5 (2.5)	0.32	2.0 (1.0)	1.5 (1.0)	0.51
Belching	1.0 (1.0)	1.0 (0.0)	0.10	1.0 (1.0)	1.0 (0.0)	0.02
Nausea	2.0 (1.0)	1.0 (0.0)	0.04	1.0 (1.0)	1.0 (0.0)	0.08
Early satiety	2.0 (2.0)	2.0 (3.0)	0.56	1.0 (1.0)	1.0 (0.0)	0.34
Postprandial Fullness	2.0 (3.0)	2.0 (2.8)	0.82	1.0 (1.0)	1.0 (1.0)	0.25
Total Symptom score	19.0 (9.0)	16.5 (5.0)	0.41	12.0 (3.0)	11.5 (4.5)	0.71

**Figure 3** Drinking capacity.**Figure 4** Change in Symptom score by intervention group.

DISCUSSION

In this study we tested the effect of itopride on some of the pathophysiological mechanisms attributed to the causation of symptoms in FD patients. Impaired accommodation is implied as one of the important factors considered to be associated with symptoms in FD patients. A primary objective of our study was to check whether itopride has any effect on gastric accommodation. We didn't find any effect of itopride on gastric accommodation when assessed through gastric scintigraphy as compared to placebo. This finding is in disagreement with a similar study which showed that itopride worsens the gastric accommodation^[29].

We also found that itopride didn't effect gastric emptying as assessed through ¹³C labeled octanoic acid breath test. A study done in Japan showed that itopride improves gastric emptying among dyspeptic patients^[30]. On the other hand a cross over study that was also done in Japan showed that itopride does not improve gastric emptying^[31]. Our study also found that itopride did not improve the drinking capacity as assessed through satiety drinking capacity test as compare to the placebo. This finding is in line with the similar findings in another study where it was found that itopride failed to improve the nutrient drink test induced symptoms^[29].

Though in previous studies it was demonstrated

that itopride improves symptoms related to FD but we found that it is not true for our set of patients. Itopride failed to show improvement in the overall symptom score as well as effect on individual symptoms including early satiety and postprandial fullness as opposed to the conclusion of a recent meta-analysis^[23]. Through previous studies we know that to achieve a considerable improvement in the symptoms of one patient, we need to treat six patients of FD^[32]. Itopride was efficacious in reducing the symptom score in Chinese patients having FD^[33]. Small sample size might be the reason which resulted in our inability to detect an improvement in individual symptoms as a result of itopride usage as compared to placebo. Our sample included younger individuals and therefore could not study the effect of itopride in older patients with FD. Lack of variability in the age might have affected the results.

Itopride is advocated for the treatment of FD as it is safer drug as compare to other prokinetic agents. In our study we found that itopride didn't prolong the QT interval compared to placebo. Similarly itopride did not raise the prolactin level compared placebo. Therefore we can say that though itopride did not demonstrate any effect it is safer prokinetic.

Inability of itopride to effect gastric accommodation and gastric emptying might be because of the genetic variability in the dopamine-D2 receptor subtype. TaqIA

polymorphism is one example where dopamine-D2 receptor is not fully expressed resulting in compromised functionality of this receptor^[34]. Mechanistic studies can identify the genetic factors like dopamine-D2 receptor variability which are anticipated to effect the efficacy of itopride among FD patients in our setting.

The strength of our study was that we used objective measures, *i.e.*, gastric scintigraphy and ¹³C labeled octanoic acid breath test to measure gastric accommodation and gastric emptying. Diagnosis of FD was based on ROME III criteria and was done after using extensive investigations to rule out organic cause for the symptoms. The study was conducted at one center and therefore we couldn't capture a broad spectrum of patients suffering from FD. We only checked the effect of 150 mg of itopride on gastric functions and symptoms. We didn't use an objective measure to find out the absorbed amount of drug in the body.

We found no effect of itopride on gastric accommodation, gastric emptying and maximum tolerated volume in patients with FD in our study.

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COMMENTS

Background

Functional dyspepsia (FD) is defined as the presence of symptoms thought to originate in the gastro-duodenal region in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms. Pharmacological treatments for patients with FD remain unsatisfactory. Itopride is a dopamine (D2) antagonist with acetylcholinesterase inhibitory actions. This agent is currently indicated for patients with various upper gastrointestinal (GI) symptoms. The anti-dopaminergic effects of itopride are truly "peripheral". There is a need to determine the effect of itopride on gastric function and to elaborate further the understanding on the basis of potential therapeutic benefit of this agent in FD patients. Through this study the authors wanted to find the effect of itopride on gastric accommodation, gastric emptying and drinking capacity in patients with FD in Pakistani population.

Research frontiers

Data related to the treatment of FD in Pakistani population is lacking. This study focused effect of itopride on gastric functions among patients with FD in their population.

Innovations and breakthroughs

Through this study the authors found out that there is no effect of itopride on gastric functions among patients of FD.

Applications

Itopride might not be a suitable medicine for treating patients with FD.

Peer-review

This is an original study investigating itopride in FD and showing no effect of it on physiological and clinical parameters.

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Randomized Clinical Trial

Role of clinical pathway in improving the quality of care for patients with faecal incontinence: A randomised trial

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Abstract

AIM

To assess the development and implementation of the Integrated Rapid Assessment and Treatment (IRAT) pathway for the management of patients with fecal incontinence and measure its impact on patients' care.

METHODS

Patients referred to the colorectal unit in our hospital for the management of faecal incontinence were randomised to either the Standard Care pathway or the newly developed IRAT pathway in this feasibility study. The IRAT pathway is designed to provide a seamless multidisciplinary care to patients with faecal incontinence in a timely fashion. On the other hand, patients in the Standard Pathway were managed in the general colorectal clinic. Percentage improvements in St. Marks Incontinence Score, Cleveland Clinic Incontinence Score and Rockwood Faecal Incontinence Quality of Life Scale after completion of treatment in both groups were the primary outcome measures. Secondary endpoints were the time required to complete the management and patients' satisfaction score. χ^2 , Mann-Whitney-*U* and Kendall tau-c correlation coefficient tests were used for comparison of outcomes of the two study groups. A *P* value of 0.05 or less was considered significant.

RESULTS

Thirty-nine patients, 34 females, consented to participate. Thirty-one (79.5%) patients completed the final assessment and were included in the outcome analysis.

There was no significant difference in the quality of life scales and incontinence scores. Patients in the IRAT pathway were more satisfied with the time required to complete management ($P = 0.033$) and had stronger agreement that all aspects of their problem were covered ($P = 0.006$).

CONCLUSION

Despite of the lack of significant difference in outcome measures, the new pathway has positively influenced patient's mindset, which was reflected in a higher satisfaction score.

Key words: Pathway; Fecal incontinence; Quality improvement

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Core tip: Critical pathways and process mapping methodology was used in industry since the 1950s and in medical field since the 1980s. This randomised trial describes the implementation of the Integrated Rapid Assessment and Treatment pathway, that was designed to provide a seamless multidisciplinary care to patients with faecal incontinence in a timely fashion, and compares it to the current standard of care. Although, there was no significant difference in quality-of-life and incontinence scores after completion of management, the new pathway positively influenced patient's mindset, as shown by the higher satisfaction scores. This is likely to reflect the structured support and thorough education patients in this group received.

Hussain ZI, Lim M, Stojkovic S. Role of clinical pathway in improving the quality of care for patients with faecal incontinence: A randomised trial. *World J Gastrointest Pharmacol Ther* 2017; 8(1): 81-89 Available from: URL: <http://www.wjgnet.com/2150-5349/full/v8/i1/81.htm> DOI: <http://dx.doi.org/10.4292/wjgpt.v8.i1.81>

INTRODUCTION

Critical pathways and process mapping methodology was used in industry, particularly in the field of engineering from as early as the 1950s. In the 1980s, clinicians in the United States began to develop the pathway tools and tried to re-define the delivery of care and attempted to identify measurable outcomes. Developed and used initially for the purpose of cost containment, in the United Kingdom in the late 1980s, the emphasis has been to use clinical pathways as a quality tool^[1].

The initial focus was to reduce length of stay (LOS) with an emphasis on nursing care^[2]. Originally, critical pathways began with admission and ended with discharge from the hospital. Today, they are usually interdisciplinary in focus, merging the medical and nursing

plans of care with those of other disciplines, such as physical therapy, nutrition, or mental health. They provide opportunities for collaborative practice and team approaches that can maximize the expertise of multiple disciplines^[1].

Goals of pathways include: (1) defining standards for expected LOS and for use of specific tests and treatments; (2) giving all team members a plan and specific roles; (3) decreasing nursing and physician documentation burdens; (4) providing a framework for collecting data; and (5) educating and involving patients and families in their care; and (6) provide better care through a mechanism that is able to coordinate clinical processes and to reduce unjustified variations and, ultimately, costs^[2,3].

Clinical pathways have four main components^[4], these are a timeline, categories of care or activities, intermediate and long term outcome criteria and variance record to allow deviations to be documented and analysed.

Here we describe the development and implementation of the Integrated Rapid Assessment and Treatment (IRAT) Pathway in the management of patients with faecal incontinence and report the outcome of a feasibility study.

MATERIALS AND METHODS

Study design

A randomised controlled trial of patients in single centre.

Patients

Adult patients referred from primary care for management of faecal incontinence in York Teaching Hospital were prospectively recruited. Following patients' initial referral, Invitation Letter and Patient Information Sheet were sent to all potential participants. Patients were then contacted by phone by the principal investigator to discuss any query they may have and obtain initial verbal consent prior to the written informed consent that was obtained on the first clinic visit.

Objectives and end points

Primary endpoints: Percentage improvement in Faecal Incontinence Scores and Rockwood Faecal Incontinence Quality of Life Scales Faecal Incontinence Quality of Life Scale (FIQoLS).

Secondary endpoints: Time scale required to achieve full assessment and management of patients in each study group. Two periods of times were calculated; time from referral by primary care to first clinic appointment and time from initiation of management, *i.e.*, first clinic appointment to completion of management; patient satisfaction.

Randomisation: Consenting patient who chose to participate in this study were randomised to either the IRAT

pathway or the Standard Care pathway. Randomisation took place by mean of Sealed Envelope Randomisation Technique. Randomisation was performed by the Hull York Medical School Statistical Consultancy service in line with the York Hospital's Standard Operating Procedure. Patients were informed about the results of randomisation by post together with the clinic appointment letter.

Sample size: This is a feasibility study. A sample size of forty patients was arbitrarily chosen conduct the study.

Ethical consideration: This study was approved by The North and East Yorkshire Alliance Research and Development Unit and the NRES Committee of the Yorkshire and the Humber Research Ethics Office. The REC reference number is 10/H1304/27.

The pelvic floor assessment pathway form

The pelvic floor assessment pathway (PFAP) Form was developed, in cooperation with Clinical Effectiveness Team, in order to construct a data base for all participants in this study. It comprises two parts "one" and "two", consisting of four (1.a, 1.b, 1.c and 1.d) and three (2.a, 2.b and 2.c) divisions respectively. Part 1 of the PFAP is concerned with documenting demographic data, medical and obstetric history, baseline St. Marks and Cleveland Faecal Incontinence Scores, baseline Rockwood FIQoLS, quality of life Visual Analogue Scale, in addition to questionnaires specific to assessment of faecal incontinence in line with NICE Guidelines recommendations. It also documents the results of anorectal laboratory studies (anorectal manometry, endoanal ultrasound, rectal compliance and anorectal mucosal electrosensitivity) in addition to any further investigation or assessment that might be required for managing individual patients. Part 2 of the PFAP documents patients' management and monitors their progress and outcome. Patients' outcome is assessed using similar assessment tools to those used in part 1, *i.e.*, FIQoLS, St. marks incontinence score (SMIS) and cleveland clinic incontinence score (CCIS) in addition to patient satisfaction and feedback score. The later comprises 9 questions that cover patients' perception of variance aspects of their management, including waiting time from referral to first clinic appointment, time required for completion of management, adequacy of time given to the patient, protection of patient's privacy and the overall quality of care in addition to feedback about the PFAP form questionnaire itself. The patients were asked to rate these various aspects of care on a scale of 1 to 5, 1 being "strongly disagree" and 5 being "strongly agree".

CCIS

Developed in 1993, the CCIS^[5] is probably still the most widely used FI severity scoring system. It gives a total score for the severity of the incontinence ranging

between 0-20; where 0 represent full continence while 20 represent the worst possible incontinence. The CCIS comprises five questions accounting for incontinence to solid stool, liquid stool and flatus in addition to the use of protective pads and change in lifestyle. Each question is scored according to the frequency of occurrence of the symptom from 0 (never) - 4 (daily). This scoring system is simple and easy to understand and formed the base of almost all subsequent FI scoring systems that are currently used.

SMIS

In addition to the five questions composing CCIS, St Mark's Score^[6] introduced an assessment of the ability to defer defecation, an additional score for the use of antidiarrhoeal medication and reduced the emphasis on the need to wear a pad. This scoring system comprises seven questions, each question is scored according to the frequency of occurrence of the symptom from 0 (never) - 4 (daily). The total score ranges between 0-24, where 0 indicates full continence while 24 represents the worst possible incontinence.

Rockwood faecal incontinence quality of life scale

Faecal Incontinence Quality of Life Scale^[7] measures specific quality of life issues expected to affect patients with faecal incontinence. It is derived from a 29 item questionnaire comprising four domains; lifestyle, coping/behaviour, depression/self-perception and embarrassment. Each domain ranges from 1 to 4; with 1 indicating a lower functional status of quality of life.

The IRAT pathway

IRAT Pathway is designed to provide a seamless multi-disciplinary care to patients with faecal incontinence in a timely fashion. Patients referred from primary care are assessed and managed by a team of surgeons, pelvic floor physiotherapist, anorectal physiology nurse practitioner and an independent researcher. Each step in patient assessment and management "event" takes place according to a preconceived timetable.

To achieve the goals of the IRAT pathway, a specialised IRAT clinic was introduced where patients are seen and assessed jointly by a colorectal surgeon with special interest in the management of faecal incontinence, pelvic floor physiotherapist and a colorectal research fellow to assess and document patient progress. This clinic takes place once every 8 wk.

Events in the IRAT pathway: Participant randomised to IRAT pathway are asked to complete part 1.a. of the PFAP before attending the first IRAT clinic; week 1: Patients are seen in IRAT clinic by surgeons and physiotherapist, completing part 1.b of PFAP; week 3: Patients undergo assessment in the Anorectal Physiology Laboratory, Part 1.c of PFAP is completed by the patients and Part 1.d. of PFAP is completed by the nurse practitioner; between week 4-week 7:

Table 1 Demographic data of patients included in analysis

Pathway	No. of patients	BMI Median (IQR)	Age Median (IQR)	Sex	
Standard care pathway	16	26.8 (23.0-31.9)	70.5 (60.0-76.0)	Female	14
				Male	2
IRAT	15	27.7 (22.8-35.8)	66.0 (59.0-77.0)	Female	12
				Male	3
P value		0.77	0.6	0.57	

IRAT: Integrated Rapid Assessment and Treatment.

All patients undergo assessment by the pelvic floor physiotherapist for suitability of biofeedback; week 8: A second IRAT clinic visit takes place for reassessment and management plan based on anorectal physiology studies and clinical and biofeedback assessments, using part 2.a of PFAP; week 16: Follow-up after completion of management.

Events in the standard care pathway: Participant randomised to Standard Care Pathway are asked to complete part 1.a. of the PFAP before attending the first clinic; patients are seen in a colorectal clinic by colorectal surgeon, completing part 1.b of PFAP; patients are assessed and treated according to the surgeon's clinical judgment. All management options available to patients in the IRAT pathway are also available to the Standard Clinic Pathway patients, including biofeedback, surgical intervention, *etc.* After completion of management, all patients, in both study arms, were asked to complete part 2.b. (final assessment) and 2.c. (patient satisfaction and feedback) of the PFAP for comparison of outcome. A reminder, by post, was sent to those who did not return the completed part 2.b. and 2.c. forms in a median of 2 mo.

Anorectal physiology laboratory assessment: Anal manometry study variables were obtained using an eight-channeled solid-state transducer catheter (Flexilog 3000, Oakfield Instruments Ltd, Evensham, Oxon, United Kingdom) using a continuous "pull through" technique. Manometric data were analysed using commercial software (Flexisoft III, Oakfield Instruments Ltd, Evensham, Oxon, United Kingdom). This included calculation of the maximum mean resting pressure, maximum mean squeeze pressure, resting (rVV), and squeeze (sVV), vector volumes, asymmetry index, and resting and squeeze vectorgrams. In addition data from endoanal ultrasound (EAUS), rectal compliance, measured by threshold rectal volume and maximum rectal volume, and rectal mucosal electrosensitivity studies were included. EAUS was performed using a standard 2D 10 MHz probe (BandK, Denmark). Colonic imaging was also performed where indicated.

Statistical analysis

Data were assessed using Microsoft Excel Spreadsheet

Table 2 Detailing obstetric history and concurrent urinary incontinence in patients included in analysis

Pathway	Vaginal delivery	Difficult labour	Perineal tear	Forceps delivery	Concurrent urinary incontinence	symptoms of global pelvic floor weakness
Standard care pathway	14/14	10	9	6	13	9
IRAT	12/14	9	8	4	9	6
P value	0.21	0.32	0.26	0.36	0.18	0.17

IRAT: Integrated Rapid Assessment and Treatment.

(Microsoft Corporation, Seattle, WA, United States) and statistical analysis was performed using SPSS v14.0 (SPSS Inc., Chicago, IL, United States). The χ^2 test was used to compare categorical variables (sex, number of deliveries, perineal tear, long labour and episiotomy, EAUS findings). The Mann-Whitney *U* test was used to compare continuous variables, including demographic data, anorectal physiology studies, time periods and the Rockwood FIQoLS. Kendall tau-c rank correlation coefficient was used to compare SMIS, CCIS and patient satisfaction score. *P* values of 0.05 or less was considered significant.

RESULTS

A total of 43 eligible patients invited to participate in this study over a period of 18 mo. Thirty-nine patients, 34 females, consented to participate. Median (IQR) age was 65 (55-75) years. Of those, 20 patients were randomised to the IRAT pathway and 19 patients were randomised to the Standard Care Pathway. Flow diagram of progress through the phases of the study is detailed in Figure 1. The median (IQR) time period from referral by primary care to first clinic appointment in our department was 5 (3-6) wk and 6 (4-8) wk for the Standard Care Pathway and the IRAT pathway respectively. The median (IQR) time period from initiation of management, *i.e.*, first clinic appointment, to competition of management, *i.e.*, discharge back to primary care was 4.5 (4-7) mo and 4 (2-6) mo for the Standard Care Pathway and the IRAT pathway respectively.

One patient withdrew from the IRAT pathway arm of this study because of resolution of her symptoms and declined further assessment. Another patient withdrew from the Standard Care Pathway without stating the reason. Of the initial 39 patients recruited in the study, 31 (79.5%) patients completed their final assessment (part 2.b) and patient satisfaction/feedback (part 2.c) components of the PFAP form. Only data from those 31 patients was included in our analysis (Figure 1).

Demographic data (age, sex, BMI) and medial and obstetric history (history of urinary incontinence, history or symptoms of pelvic floor weakness, history of vaginal delivery, difficult labour, perineal tear and forceps

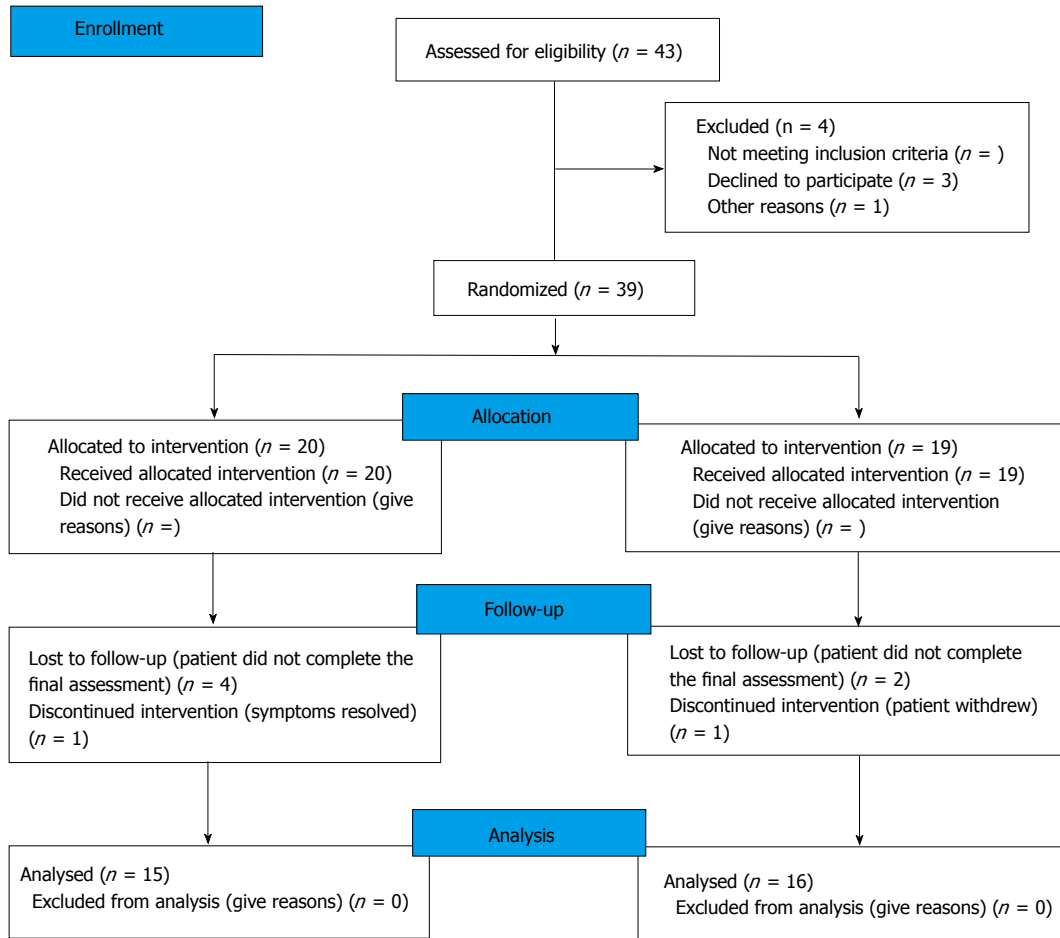


Figure 1 Flow diagram of progress through the phases of the study.

delivery) of those patients are detailed in Tables 1 and 2 respectively.

There was no significant difference in demographic data, obstetric history and anorectal laboratory test results (Table 3) between the two groups of this study. Similarly, there was no significant difference in baseline FIQoLS, SMIS and CCIS between the two study groups (Tables 4 and 5).

Three patients in Standard Care Pathway underwent perianal injection of bulking agent (Permacol®), one of them subsequently referred to SNS in a tertiary care centre due to persistence of symptoms. Another patient in the Standard Care Pathway was referred to the gynaecology team with severe uterine prolapse and subsequently underwent hysterectomy. One patient in the IRAT pathway was referred for SNS a tertiary care centre. The rest of the patients in both study groups were managed conservatively, mainly with pelvic floor exercise and biofeedback. One patient's symptoms resolved after amending his cholesterol medication.

Final follow-up with FIQoLS, SMIS, CCIS and patient satisfaction score was carried out in a median (IQR) of 1 (1-3) mo after completion of management. This shows no significant difference in any of the four scales of FIQoLS, *i.e.*, the lifestyle, coping, depression and embarrassment scales, between both study groups

(Table 6). Similarly there was no difference in CCIS or SMIS at final follow-up (Table 7).

Patients' satisfaction scores in 7 of the 9 item questionnaire were not significantly different (Table 8). However patients in the IRAT pathway were more satisfied with the time required for completion of treatment (from first clinic appointment to discharge) than those in the Standard Care Pathway ($P = 0.033$). There was also a stronger agreement among the IRAT Pathway group that the questionnaire in the FPAP covered all aspects of their problem ($P = 0.006$).

The median (IQR) time period from referral by primary care to first clinic appointment was similar at 5 (3-7) wk for the both Standard Care Pathway and the IRAT pathway ($P = 0.889$). The median (IQR) time period for completion of management was 4.5 (4-7) mo and 4 (2-5) mo for the Standard Care Pathway and the IRAT pathway respectively. This was not significantly different ($P = 0.307$).

DISCUSSION

This study shows no significant difference in outcome measures such as FIQoLS, SMIS and CCIS when patients were managed in the IRAT Pathway compared to the Standard Care Pathway. The IRAT Pathway was

Table 3 detailing anorectal laboratory test results in patients included in the analysis

Anorectal physiology variables	IRAT pathway Median (IQR)	Standard care pathway Median (IQR)	P value
MMRP	46.0 (36.0-80.0)	55 (38.5-72)	0.96
MMSP	74.0 (57.0-89.0)	50.0 (37.0-72.0)	0.88
Resting victor volume	33308.0 (16559.2-54994.0)	51224.0 (29444.0-77663.0)	0.17
Squeeze victor volume	61168.0 (44393.0-165403.0)	81303 (51751.0-118808.5)	0.79
Squeeze asymmetry	29.7 (11.7-27.1)	14.4 (8.4-16.9)	0.07
Resting asymmetry	20.9 (13.5-31.0)	17.9 (11.2-27.1)	0.41
USS-IAS	2 abnormal	2 abnormal	1.00
USS-EAS	2 abnormal	1 abnormal	0.59
Resting vectrogram	4 abnormal	5 abnormal	0.94
Squeeze vectrogram	3 abnormal	5 abnormal	0.43
TRV	85 (50-100)	80 (50-95)	0.85
MRV	140 (100-195)	140 (100-195)	0.94
AME (high)	6.5 (5.2-10.6)	7.1 (5.5-11.3)	0.93
AME (mid)	5.3 (3.6-7.5)	5.9 (4.6-7.7)	0.89
AME (low)	4.7 (2.8-6.6)	5.1 (3.0-6.5)	0.85

MRV: Maximum rectal volume; TRV: Threshold rectal volume; MMRP: Maximum mean resting pressure.

Table 4 Comparison between baseline rockwood faecal incontinence quality of life scales of both study groups

Baseline	FIQoLS 1 Median (IQR)	FIQoLS 2 Median (IQR)	FIQoLS 3 Median (IQR)	FIQoLS 4 Median (IQR)
IRAT pathway	3.6 (2.0-2.4)	2.7 (1.4-3.4)	3.7 (2.3-4.1)	2.7 (1.3-3.8)
Standard care pathway	3.5 (2.3-3.7)	2.4 (1.6-3.0)	3.1 (2.0-3.7)	2.0 (1.3-2.7)
P value	0.44	0.94	0.11	0.22

IRAT: Integrated Rapid Assessment and Treatment; FIQoLS: Faecal Incontinence Quality of Life Scale.

Table 5 Comparison between baseline St. marks incontinence score and cleveland clinic incontinence score of both study groups

Baseline	CCIS Median (IQR)	SMIS Median (IQR)
IRAT pathway	8.0 (33.5-11.5)	13.0 (5.5-13.0)
Standard care pathway	9.5 (5.0-15.0)	12.0 (7.0-16.0)
P value	0.11	0.18

IRAT: Integrated Rapid Assessment and Treatment; CCIS: Cleveland clinic incontinence score; SMIS: St. marks incontinence score.

Table 6 Comparison between Rockwood Faecal Incontinence Quality of Life Scales of both study groups after completion of management

After completion of management	FIQoLS 1 Median (IQR)	FIQoLS 2 Median (IQR)	FIQoLS 3 Median (IQR)	FIQoLS 4 Median (IQR)
IRAT pathway	3.9 (2.2- 4.0)	2.9 (1.8 3.8)	3.9 (2.3-4.1)	3.0 (1.8-3.8)
Standard care pathway	3.6 (2.4-4.0)	3.8 (1.7-4.0)	3.5 (2.1-3.9)	2.3 (1.6-3.7)
P value	0.51	0.92	0.18	0.87

IRAT: Integrated Rapid Assessment and Treatment; FIQoLS: Faecal Incontinence Quality of Life Scale.

Table 7 Comparison between St. marks incontinence score and cleveland clinic incontinence score of both study groups after completion of management

After completion of management	CCIS Median (IQR)	SMIS Median (IQR)
IRAT pathway	6.0 (1.5 -11.5)	7.0 (30-15.5)
Standard care pathway	7.5 (3.0-12.0)	9.5 (4.0-11.0)
P value	0.37	0.85

IRAT: Integrated Rapid Assessment and Treatment; CCIS: Cleveland clinic incontinence score; SMIS: St. marks incontinence score.

designed to expedite the management of patients with FI. The IRAT clinic takes place once every 8 wk. During the time periods between first and second and second and third clinic visits, the patient would have completed their assessments and treatment respectively. However, this study shows that there was no significant difference in the waiting time for the first clinic appointment and in the time required for completion of management between the two study groups. This could well be due to the inflexibility of the preconceived timetable in the IRAT Pathway. When patients have asked to postpone or change their clinic dates for various reasons, which

occurred in the case of 4 patients in the IRAT Pathway, they had to wait for another 8 wk for the next clinic appointment. The Standard Care Pathway, on the other hand, was more flexible, and since colorectal clinics take place every week, they could accommodate for patients' cancelations and appointment changes on weekly basis. By the same token, patient factors and preferences may have influenced these time scales. This is reflected in the patient satisfaction questionnaire, where patients in the IRAT pathway were more satisfied with the time required for completion of management, in spite of the lack of significant difference in the time scale itself.

Table 8 Comparison of patient satisfaction score between the integrated rapid assessment and treatment and the standard care pathways

Please rate your degree of satisfaction with each of the following aspect	Standard care pathway median (IQR)	IRAT pathway median (IQR)	P value
The waiting time from seeing your GP until been seen at York hospital was acceptable	4 (3-4)	4 (4-5)	0.07
The waiting time from being seen at York Hospital until completing your treatment was acceptable	4 (3-4)	4 (4-5)	0.03
The questions you were asked to complete were relevant to your problem?	4 (4-4)	4 (4-5)	0.24
The questions you were asked to complete were clear and easy to answer?	4 (4-4)	4 (4-5)	0.28
The questions you were asked to complete covered all aspect of your problem?	4 (3-4)	4 (4-5)	0.01
You were supported and given clear advices/instructions throughout management	4 (4-4)	4 (4-5)	0.08
You were given enough time to explain your problem/concerns	4 (4-4)	4 (4-5)	0.08
Your privacy and dignity were respected throughout management	4 (4-5)	4 (4-5)	0.43
The over all quality of care you received was high	4.5 (4-5)	4 (4-5)	0.85

IRAT: Integrated Rapid Assessment and Treatment.

Patients in the IRAT Pathway also had stronger agreement that all aspects of their problem were addressed. This could reflect the structured support and thorough education that patients in this group received along with interaction with pelvic floor and biofeedback therapists both in the clinic and in the laboratory.

Both study groups have rated the overall quality of care equally, which, in addition to a non-significantly different outcome measures (FIQoLS, CCIS and SMIS), means the introduction of the IRAT Pathway did not have a major impact on the quality of patient care.

In spite of the outcome measures of this study, patient satisfaction seemed to increase with the use of the IRAT pathway. This finding is compatible with outcomes of other similar studies. Lawson *et al*^[8] report that patient and parent satisfaction increased because of the promptness of securing discharge prescriptions. Goode^[9] discovered that patients who had a care map and a nurse case manager were more satisfied with their care.

There is evidence that pathways are more likely to be effective when applied to conditions and procedures with lower severity/complexity of illness, high volume and higher length of stay^[10]. This does not apply to FI which is a multifactorial condition with complex aetiology. In addition the volume of patient referred our department for management of FI was relatively low. The risk of "contamination" of the control sample, *i.e.*, communication between experimental and control professionals, was not considered in this study, especially that some of the Standard Care Clinic were run by the same colorectal consultant conducting the IRAT Clinics. Some or all of these factors could have contributed to the final outcome of this study.

Clinical pathways applied to patients with a cardiovascular disease showed a tendency towards a decreased treatment variation, improved guideline compliance and reduced costs. However, the evidence of the effectiveness of clinical pathways in cardiovascular medicine can not be generalized because of the insufficient number of controlled studies^[11]. There was a strong decline in both the average length of stay and its

variation after implementation of CP in inguinal hernia repair^[3]. Similar finding were observed in knee and hip arthroplasty procedures^[12]. However, no significant difference in patient outcomes was seen.

On the other hand, no benefit of using clinical pathway in stroke patients was detected over conventional multidisciplinary care^[10,13,14]. Functional recovery was faster and quality of life outcomes better in patients receiving conventional multidisciplinary care. Some studies reported major failures in implementation of clinical pathways for stroke and their implementation was discontinued^[3].

Some studies did suggest that the use of clinical pathways had no influence on patient-care outcomes, by the same token they also stated that there was no evidence at all that they had any negative effect^[15]. However, no, few, or even negative results after implementing CP hardly ever get published^[15].

How health care should respond to clinical pathways that have not been shown to improve care, such as some the pathways for strokes and renal failure^[3] is not clear and further research is needed to answer this question^[16]. The answer depends on the risks, costs, and opportunity costs of continuing to implement critical pathways or other strategies^[16].

It has been assumed that critical pathways are not associated with risk, although there are relatively few studies to support or refute that belief. However, critical pathways might be costly to develop, update, and implement. There may also be opportunity costs of not pursuing other strategies that might more effectively improve quality, reduce costs, and enhance patient safety, since these other strategies must compete for organizational resources^[16].

Despite widespread enthusiasm for critical pathways, rigorous evidence to support their benefits in health care is extremely limited. However, understanding what evidence-based information is, and translating this information into practice using reminder systems or other effective implementation strategies, can potentially improve care, reduce costs, and enhance safety^[16-20].

Rigorous evaluation of CP and medical management

approaches is essential in order to determine the effectiveness of CP in particular area of medical care. Pearson *et al.*^[21] reported significant reductions in lengths of stay after implementation of CP for surgical conditions. However, this reduction in LOS was similar to those at health care organizations at which there were no organized CP efforts in place. The CP program was responsible for very modest improvements in patient care, and was probably without a measurable "return on investment." These results occurred in an organization where the investigators are extremely knowledgeable and experienced in the field of critical pathways^[22]. Only after the authors observed declining lengths of stay in organizations without critical pathways did they believe that the reductions at their organization were more likely to be a result of secular trends rather than the critical pathways^[16]. In this study we randomised patients between CP and standard care which has given us the advantage to overcome this confounding factor. The findings in this study are, however, consistent with those from Pearson *et al.*^[21] study.

Studies should also determine the clinical and financial return on investment of these efforts. Organizations should identify which components of their current clinical quality improvement efforts are effective, and which are not. For strategies that are without measurable benefit, consideration should be given to learning from those experiences and may be redirecting resources to more effective quality improvement strategies^[16].

Finally, in spite of the lack of significant difference in outcome measures, the IRAT Pathway has positively influenced patient's mindset, which was reflected in a higher satisfaction score. This has an important impact on the overall care for patients with problems such as faecal incontinence.

COMMENTS

Background

The management of faecal incontinence is widely varied, ranging from conservative management with dietary modification, medications and behavioral interventions to invasive therapy including complex surgery. No previous study has discussed the role of clinical pathway in the management of faecal incontinence.

Research frontiers

There is evidence that clinical pathways applied to patients with certain conditions, such as cardiovascular disease, showed a tendency towards a decreased treatment variation, improved guideline compliance and reduced costs. However, this evidence cannot be generalized to other conditions, such as faecal incontinence, because of the insufficient number of controlled studies

Innovations and breakthroughs

This is the first randomized controlled study to evaluate the development and implementation of clinical pathway in the management of patients with faecal incontinence and measure its impact on patients' care.

Applications

This pilot study's design and findings could be used to determine sample size for a larger randomised controlled study aiming to test the impact of clinical pathway and structured patient support and thorough education on clinical outcome and

satisfaction in patients with faecal incontinence.

Terminology

Critical pathways and process mapping methodology was used in industry, particularly in the field of engineering from as early as the 1950s. In the 1980s, clinicians in the United States began to develop the pathway tools and tried to re-define the delivery of care and attempted to identify measurable outcomes. Developed and used initially for the purpose of cost containment, in the United Kingdom in the late 1980s, the emphasis has been to use clinical pathways as a quality tool.

Peer-review

The study is well designed, the manuscript is well written and new data have been provided.

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