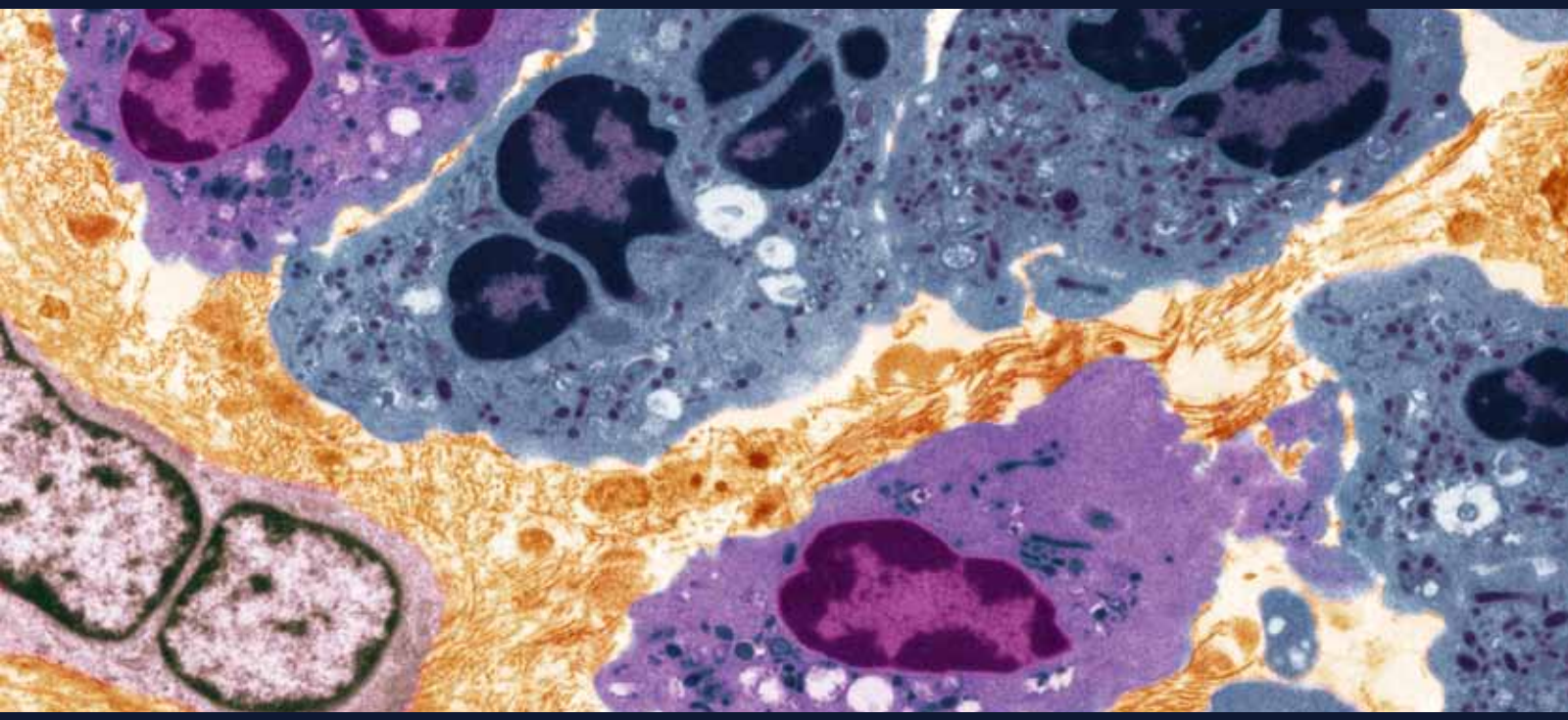
International Journal of Inflammation

# Inflammatory Bowel Disease

Guest Editors: David B. Sachar, Derek Jewell, Christoph Gasche, and Jonathan Braun



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# Editorial Board

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 341052, 2 pages
doi:10.1155/2012/341052

# *Editorial*
# Inflammatory Bowel Disease

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Received 17 January 2012; Accepted 17 January 2012



Studies of inflammatory bowel disease (IBD) over many decades have spanned a wide range of topics from clinical epidemiology and diagnosis, through dietary and pharmacologic therapy and immunologic and biologic etiology, to pathophysiologic links to malignancy. In this special issue devoted to IBD, the Guest Editors and one of the authors have selected a series of seven papers from seven countries that reflect this entire spectrum.

Leading off with clinical epidemiology, Y. Correa et al. from the University of Puerto Rico call our attention to the increasing prevalence of both ulcerative colitis (UC) and Crohn's disease (CD) among Hispanics. Particularly noteworthy is their observation that no particular phenotypic features consistently distinguish this Puerto Rican cohort from other white, non-Hispanic populations. Could we take these findings to infer that environmental factors are playing a role in the presentation of IBD that trumps or at least balances the role of genetics?

From clinical epidemiology in Puerto Rico, we move to clinical diagnosis in Italy. E. Calabrese et al. at the University of Rome offer us a review of the utility of contrast-enhanced ultrasound of the small bowel in the evaluation of patients with CD. With or without radiation, both CT and magnetic resonance (MR) enterography are relatively expensive and labor-intensive, so a lower-cost, more convenient alternative could be a welcome addition to the armamentarium. Will contrast-enhanced ultrasound fill this role?

No survey of IBD could be complete without at least some attention to therapy. G. M. Fung and A. Szilagyi from McGill University in Montreal caution us against too readily embracing the fad of carbohydrate withdrawal as dietary treatment for the symptoms of IBD. They remind us of the potential importance of carbohydrates as a substrate for bacterial production of short-chain fatty acids, which are possibly critical in maintaining anti-inflammatory homeostasis in the intestinal tract.

Another paper in this special issue shifts from dietary to pharmacologic therapy. While anti-TNF and anti-integrin molecules have been occupying most of the spotlight for IBD treatment in the past decade, novel approaches targeting other pathways will undoubtedly yet emerge. L. R. Fitzpatrick from Penn State College of Medicine calls attention to one of the key alternative pathways in his review of the IL-23/IL-17 axis. Certainly, it has been a seminal discovery that these two closely intertwined pathways can both be neutralized by targeting the common p40 subunit of IL-12 and IL-23. L. R. Fitzpatrick's review shows us that several different approaches to targeting this central inflammatory pathway, already usefully exploited in treating psoriasis, might well prove beneficial in IBD as well.

The paper by T. L. Holm et al. from Denmark moves from the bedside to the bench by employing a murine model of CD. Using the severe combined immunodeficiency disease (SCID) adoptive T-cell transfer model of colitis, these authors provide an intriguing link to L. R. Fitzpatrick's review. It turns out that, among an array of eight different agents tested for prevention or treatment of this form of experimental colitis, only anti-IL-12p40 and abatacept (cytotoxic T-lymphocyte antigen 4 immunoglobulin) induced remission of established disease. To be sure, this murine system is far from a perfect model of human IBD, but the Danish study is certainly a confirmation of the theoretical

anti-inflammatory potential of targeting the IL-12/23 pathway.

The penultimate paper concentrates on the newest candidate etiology of IBD, namely, intestinal dysbiosis. G. De Hertog et al. from Leuven, Belgium, used laser microdissection of intestinal tissues from four CD patients, six inflammatory disease controls, and three noninflamed controls to demonstrate "significant changes of the composition, abundance and location of the gut microbiome in [Crohn's] disease." This avenue of research is clearly going to be a major focus over at least the next decade.

Finally, our special issue presents a review of several inflammation-associated colorectal cancer model developed by T. Tanaka from Japan. He expresses the hope "that use of these models will advance elucidation of the mechanisms (methylation and microRNA) of inflammation-associated colorectal carcinogenesis, exploration of its suppression and mechanisms, and clarification of the mechanisms of tumor-promotion activity of DSS [dextran sodium sulfate]."

We readily acknowledge that the seven articles selected by the three indefatigable Guest Editors and me from the many manuscripts submitted will not provide definitive answers to the mysteries of IBD. Nonetheless, this special issue makes a creditable effort to identify some of the most important questions.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 389404, 8 pages
doi:10.1155/2012/389404

*Review Article*

# Novel Pharmacological Approaches for Inflammatory Bowel Disease: Targeting Key Intracellular Pathways and the IL-23/IL-17 Axis

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Received 27 September 2011; Accepted 28 November 2011

Academic Editor: Derek Jewell



This review identifies possible pharmacological targets for inflammatory bowel disease (IBD) within the IL-23/IL-17 axis. Specifically, there are several targets within the IL-23/IL-17 pathways for potential pharmacological intervention with antibodies or small molecule inhibitors. These targets include TL1A (tumor necrosis factor-like molecule), DR3 (death receptor 3), IL-23, IL-17 and the receptors for IL-23 and IL-17. As related to IBD, there are also other novel pharmacological targets. These targets include inhibiting specific immunoproteasome subunits, blocking a key enzyme in sphingolipid metabolism (sphingosine kinase), and modulating NF-$\kappa$B/STAT3 interactions. Several good approaches exist for pharmacological inhibition of key components in the IL-23 and IL-17 pathways. These approaches include specific monoclonal antibodies to TL1A, IL-17 receptor, Fc fusion proteins, specific antibodies to IL-17F, and small molecule inhibitors of IL-17 like Vidofludimus. Also, other potential approaches for targeted drug development in IBD include specific chemical inhibitors of SK, specific small molecule inhibitors directed against catalytic subunits of the immunoproteasome, and dual inhibitors of the STAT3 and NF-$\kappa$B signal transduction systems. In the future, well-designed preclinical studies are still needed to determine which of these pharmacological approaches will provide drugs with the best efficacy and safety profiles for entrance into clinical trials.

## 1. Introduction

During the past decade, there has been an expansion in new scientific knowledge related to the pathogenesis of inflammatory bowel disease (IBD). This knowledge has been summarized rather recently in published reviews, which provided key insights into IBD pathogenesis [1, 2]. Briefly, IBD consists of two distinct diseases, Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are thought to arise due to a combination of genetic variations and alterations in the bacterial flora, which can subsequently drive a dysregulated immune response that results in chronic intestinal inflammation [1, 2].

Recent information related to the pathogenesis of IBD has provided the rationale for new pharmacological approaches to better treat the intestinal inflammation and related symptoms in patients. Another scientific review has succinctly summarized current therapies for IBD: mesalazine-based drugs, corticosteroids, immunosuppressive drugs (azathioprine/6-mercaptopurine, methotrexate, cyclosporin, anti-TNF agents), as well as emerging biologic agents such as antiadhesion and antiintegrin molecules [3].

This review will primarily focus on possible pharmacological targets within the IL-23/IL-17 proinflammatory pathway (i.e., IL-23/IL-17 Axis), including some work from our laboratory [4]. Secondarily, this review will provide insights into some other novel pharmacological targets, such as inhibiting specific immunoproteasome subunits, blocking a key enzyme in sphingolipid metabolism (sphingosine kinase), and modulating NF-B/$\kappa$STAT3 interactions. Scientific data supporting these pharmacological targets will be provided from the published literature [5–12].

There are several targets within the IL-23/IL-17 pathways for potential pharmacological intervention with antibodies or small molecule inhibitors. These targets include TL1A, DR3, IL-23, IL-23R, IL-17, and IL-17R (Figure 1).

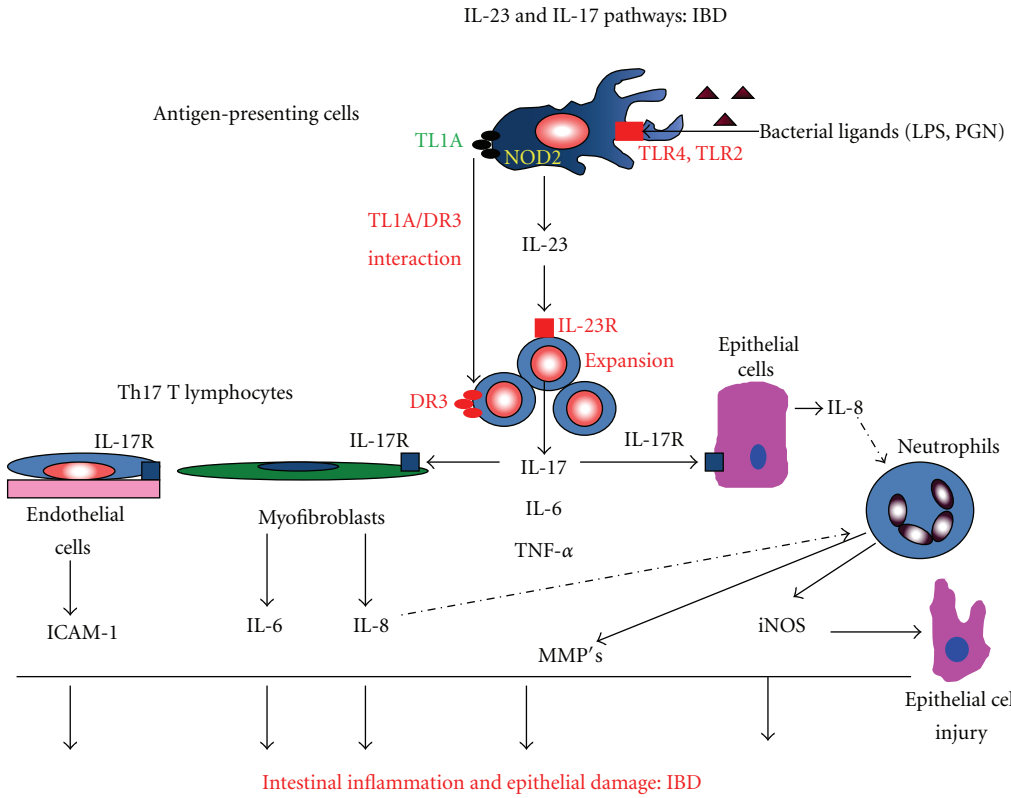


Figure 1: This figure shows relevant cell types, mediators, and potential pharmacological targets associated with IL-23 and IL-17 pathways (IL-23/IL-17 Axis), which are operative within the context of inflammatory bowel disease (IBD). Bacterial ligands (lipopolysaccharide [LPS] and peptidoglycan [PGN]) bind to their respective toll-like receptors (TLR4 and TLR2) and induce IL-23 release from antigen-presenting cells (APC's). IL-23 binds to the IL-23 receptor (IL-23R) to stimulate expansion of Th-17-producing cells, which release IL-17. In addition, interactions between TL1A (tumor necrosis factor-like molecule) on APC's and DR3 (death receptor 3) on T lymphocytes induces the secretion of IL-17. These pathways also promote the secretion other proinflammatory cytokines like IL-6 and TNF-$\alpha$. IL-17 stimulates the expression of adhesion molecules (e.g., ICAM-1) on endothelial cells, as well as the release of IL-6 and IL-8 from myofibroblasts and epithelial cells. IL-8 acts as a chemotactic factor for neutrophil influx into the intestine. Infiltrating neutrophils release inflammatory mediators like matrix metalloproteinases (MMP's) and inducible nitric oxide synthase (iNOS). This sequelae of pathogenic events leads to the chronic inflammation and epithelial cell damage associated with IBD.

## 2. TL1A/DR3

As shown in Figure 1, upstream binding of bacterial derived ligands such as lipopolysaccharide (LPS) and peptidoglycan (PGN) to their specific toll-like receptors (TLR4 and TLR2, respectively) can induce TL1A (tumor necrosis factor-like molecule) expression in antigen presenting cells (like dendritic cells) [13]. Downstream, the interaction of TL1A with DR3 (death receptor 3) results in the production of IL-17 from Th17 T lymphocytes [14].

The interaction between this TNF-family member (TL1A) and its receptor DR3 plays an important role in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [15]. More recently, other investigators have published an informative review on the role of the TL1A-DR3 pathway in the pathogenesis of IBD [14]. Of note, TL1A expression is increased in the inflamed intestinal tissue of patients with CD [1].

In 2008, Takedatsu and colleagues showed that TL1A and DR3 expression was upregulated in the gut-associated lymphoid tissue (GALT) of mice with chronic dextran sulfate sodium (DSS)-induced colitis [16]. Importantly, from a pharmacological standpoint, a monoclonal antibody (mAb) to TL1A effectively attenuated chronic DSS-induced colitis, as well as T-cell transfer colitis in mice [16]. This antibody also improved established chronic colitis. The anticolitis effects were associated with decreases in IFN-$\gamma$, IL-17, and IL-6 production from GALT [16]. These results clearly established targeting of TL1A, as a rational pharmacological approach for IBD. More recently, two other research groups have generated transgenic mice with enhanced expression of TL1A in T-cells or dendritic cells [17, 18]. These mice developed predominantly small intestinal pathology, which was dependent upon DR3, IL-13, and IL-17 [17, 18]. Important studies were then carried out in mice with acute trinitrobenzenesulfonic acid (TNBS)-induced colitis. These mice were treated with an antagonistic mAb to TL1A, a DR3-Fc fusion protein, or an antagonistic mAb to DR3 [17]. Mice treated with anti-TL1A showed a marked improvement in indices of TNBS-induced colitis. Also, partial protection

against this murine colitis was found with the anti-DR3 pharmacological approaches [17]. Taken as a whole, these results further suggest that targeting of the TL1A-DR3 pathway could be a good pharmacological approach for both types of human IBD (CD and UC) [17–19].

## 3. IL-23

As shown in Figure 1, upon stimulation by appropriate ligands, IL-23 is produced by antigen-presenting cells. After binding to the appropriate receptor (IL-23R), this cytokine can stimulate the production of IL-17, TNF-$\alpha$, and IL-6 from T-cells. Therefore, IL-23 was proposed to play an integral role in the pathogenesis of IBD [20]. From a potential therapeutic standpoint, Elson and colleagues created T-cell transfer colitis in SCID mice recipients with bacterial reactive Th17 CD4+ T-cells [21]. Treatment of these mice with an antibody to the p19 subunit of IL-23 both prevented T-cell transfer colitis and effectively treated established colitis [21]. This is a rather specific therapeutic approach for treating IBD, because only the p19 subunit is targeted. This subunit is endogenous only to IL-23 but is not shared by IL-12, like the common p40 subunit [21]. An antibody targeting the common p40 subunit (Ustekinumab) has shown some evidence of efficacy in patients with CD (phase II a trial) and is undergoing further clinical trials [22, 23]. Ustekinumab was generally well tolerated in these IBD patients [22, 23]. In the long term, it remains to be determined whether an antibody targeting solely IL-23 p19 will have a better efficacy/safety ratio than Ustekinumab in IBD patients [21–23].

In order to investigate a downstream component of the IL-23 pathway, Takedatsu et al. determined whether a mAb to the IL-23 receptor (IL-23R) attenuated indices of acute or chronic-DSS-induced colitis in mice [16]. Interestingly, the chronic phase of colitis was attenuated by treatment with the IL-23 mAb to a greater degree than the acute phase of colonic inflammation [16]. Furthermore, the anticolitis effects with the IL-23 mAb seemed to be less dramatic than the effects with the mAb to TLA1 [16]. As suggested by the authors, it is possible that neutralizing TL1A could induce more comprehensive effects than just blocking downstream components of the IL-23/IL-17 axis (Figure 1) [14–16]. Therefore, in addition to affecting IL-17 production (Figure 1), blocking the IL-12/IFN-$\gamma$ pathway by TL1A neutralization may also be needed to effectively treat the colonic inflammation associated with human IBD [16]. Interestingly, it has recently been reported that, in CD patients, there is a population of CD $161^{(+)}$ CD4 T-cells which produce both IL-17 and IFN-$\gamma$ [24]. As a whole, these results emphasize the complexity in the pathogenesis of IBD, involving multiple inflammatory mediators. This complexity must be recognized within the context of developing novel pharmacological approaches for UC and CD.

## 4. IL-17

Elevated expression of IL-17 has been reported in the inflamed intestine of patients with UC and CD [2, 24]. IL-17, which is the prototypical cytokine produced by Th17 cells, plays a potential role in the amplification of intestinal inflammation. Specifically, IL-17 stimulates various cell types (endothelial cells, myofibroblasts, and epithelial cells) to produce proinflammatory mediators that amplify intestinal inflammation (Figure 1) [25, 26]. Therefore, it is interesting that variable and somewhat contrasting results have been obtained with approaches that inhibit the function of IL-17 in animal models of IBD [25–28]. These contrasting results could be related to different functions of IL-17A and IL-17F, within the specific context of intestinal inflammation [25–27]. In this regard, Yang and colleagues showed that murine DSS-induced colitis was worsened in IL-17A knockout (KO) mice but significantly improved in IL-17F KO mice [27]. Furthermore, a protective role was also proposed for IL-17A in a T-cell transfer model of colitis [26, 28]. In contrast, Zhang and colleagues showed that acute TNBS-induced colitis was attenuated in IL-17 receptor (IL-17R) KO mice, as well as in animals treated with an IL-17 R:Fc fusion protein [25]. It is probable that the IL-17 R KO mice would not respond to either IL-17A or IL-17F, suggesting that inhibition of both forms of IL-17 is needed for attenuation of colitis [25].

Vidofludimus (4SC-101) is a novel small molecule inhibitor of dihydroorotate dehydrogenase (DHODH), which is a key enzyme involved in pyrimidine (i.e., uridine biosynthesis) in activated lymphocytes [4]. However, our research group showed that Vidofludimus inhibited IL-17 production in activated lymphocytes, even in the presence of exogenous uridine. Our results suggested a pharmacological effect that was independent of inhibiting DHODH and T-cell proliferation [4]. Subsequently, we showed that Vidofludimus could inhibit IL-17 secretion in activated splenocytes by inhibiting STAT3 and NF-$\kappa$B-signaling pathways [29]. Importantly, Vidofludimus attenuated various parameters of acute TNBS-induced colitis in mice, including IL-17 production [4]. Specifically, this anticolitis profile was associated with a reduction in the colonic expression of both IL-17 A/A homodimers, as well as IL-17 F/A heterodimers [4]. These results suggested that Vidofludimus would be an appropriate drug for use in patients with IBD. Indeed, in a recent Phase II European clinical trial, Vidofludimus demonstrated a good efficacy and safety profile in patients with IBD [30]. Because this small molecule compound has the potential for inhibiting T-lymphocyte proliferation, as well as inhibiting relevant IL-17A and IL-17F signal transduction pathways, it is an interesting candidate for future clinical studies.

Finally, with regard to IL-17 inhibition, AIN457 (Secukinumab) is a human anti-IL-17A antibody that has been developed by Novartis Healthcare [26]. Based on an oral presentation at the 2011 Digestive Disease Week meeting, it seems that recent clinical results in CD patients treated with AIN457 have been negative. Specifically, Secukinumab-treated patients did not show improvement in parameters of disease [31]. At first glance, these results seem to be counterintuitive to the schematic pathways in Figure 1. However, plasma levels of IL-17F, as well as IL-17F production by stimulated splenocytes, are elevated in IL-17A-deficient mice [32]. In this regard, the preclinical literature suggests that

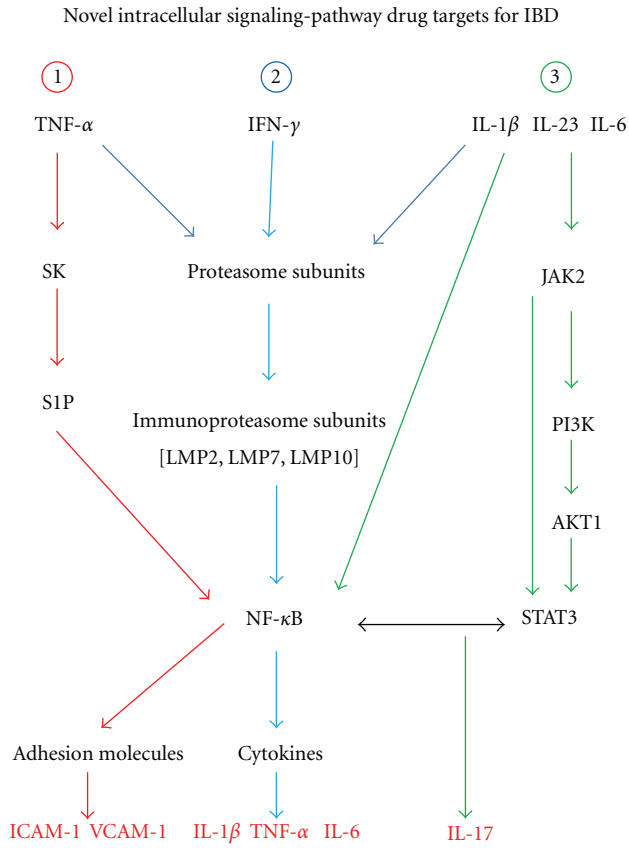


Figure 2: This figure shows three novel intracellular signaling pathways involved in the pathogenesis of IBD. Pathway 1: TNF-$\alpha$ induces adhesion molecule expression in endothelial cells, as well as proinflammatory cytokine (IL-1$\beta$, IL-6) production by monocytes, through a sphingosine kinase (SK), sphingosine-1-phosphate (S1P), nuclear factor-kappa B (NF-$\kappa$B)-dependent pathway. Pathway 2: upon stimulation of cells with proinflammatory cytokines (IFN-$\gamma$, TNF-$\alpha$, and IL-1$\beta$), constitutive proteasome subunits are converted to the immunoproteasome subunits $\beta$1i (LMP2), $\beta$2i (LMP10, MECL-1), and $\beta$5i (LMP7) [38–40]. Functionally, immunoproteasome subunits play a role in NF-$\kappa$B signaling. Pathway 3: dual activation of NF-$\kappa$B and STAT3 pathways controls the expression of IL-17. As shown in this figure, crosstalk between these three pathways occurs, thereby promoting intestinal inflammation. Specific components of these pathways such as sphingosine kinase (SK), immunoproteasome subunits (LMP2, LMP7, and LMP10), and interactions between NF-$\kappa$B/STAT3 represent possible pharmacological targets for IBD. In the figure: LMP is low molecular mass polypeptide (2, 5, or 10); JAK2 is Janus Kinase 2; PI3K is phosphoinositide-3 kinase; AKT1 is Alpha serine/threonine-protein kinase.

specifically inhibiting IL-17F, and/or inhibiting both IL-17 A and IL-17F, may be necessary to achieve good anticolitis actions [4, 25, 27].

## 5. Novel Intracellular Signaling Targets for IBD

Figure 2 shows three intracellular signaling pathways that are potentially involved in the pathogenesis of IBD: (1) altered sphingolipid metabolism, whereby the enzyme sphingosine kinase (SK) appears to play a critical role in signaling by TNF-$\alpha$ [9–11], (2) upregulation of immunoproteasome subunits by proinflammatory cytokines, which downstream is connected to activation of the NF-$\kappa$B signal transduction system [5–8, 33–35], and (3) dual activation of NF-$\kappa$B and STAT3 signal pathways by cytokines, which results in enhanced IL-17 production by leukocytes [29, 36, 37]. These pathways are summarized in Figure 2. Interestingly, as shown in this figure, crosstalk between inflammatory pathways occurs, which likely promotes intestinal inflammation.

Based on the pathways outlined in Figure 2, this section of the review will specifically focus on three pharmacological targets for IBD: (1) inhibition of SK, (2) inhibiting specific catalytic subunits of the immunoproteasome, and (3) modulating NF-$\kappa$B/STAT3 interactions.

## 6. SK Inhibition

SK is involved in the conversion of sphingosine to sphingosine-1-phosphate (S1P) [9–11]. Importantly, SK exists as two isoforms (SK1 and SK2), with diverse biological functions, which have been reviewed elsewhere [41, 42]. A critical step in the mechanism of action for TNF-$\alpha$ includes the activation of SK [9–11, 41, 42]. Of critical relevance to this review, SK signals downstream through activation of the transcription factor NF-$\kappa$B (Figure 2, pathway 1). Specifically, *in vitro* studies have shown that TNF-$\alpha$ induces adhesion molecule expression in endothelial cells, as well as proinflammatory cytokine (IL-1$\beta$, IL-6) production by

monocytes, through an SK-SIP-NF-$\kappa$B-dependent pathway (Figure 2) [9, 43, 44]. Recent results have shown that SK1 expression was increased in colonic tissue samples from patients with UC [11]. The potential role that SK plays in the generation of proinflammatory molecules relevant to the pathogenesis of IBD has prompted investigators to evaluate whether SK inhibition can effectively attenuate intestinal inflammation.

Snider et al. showed that DSS-induced colitis was less severe in SK-1-deficient ($SK1^{-/-}$) mice compared to wild-type control mice [11]. From a pharmacodynamic standpoint, intestinal SK1 mRNA expression, as well as SK activity (generation of S1P) were both attenuated in SK1-deficient mice. These results suggest that specific inhibition of SK1 may represent a valid pharmacological approach for IBD [11]. Maines and colleagues showed that treatment of mice with ABC249640 (a selective small molecule inhibitor of SK2) effectively attenuated parameters of murine DSS-induced colitis, as well as TNBS-induced colitis in mice and rats [9, 10]. Treatment of mice with ABC294640 resulted in reduced colonic S1P levels, as well as decreased levels of proinflammatory cytokines (IL-1$\beta$, IL-6, TNF-$\alpha$, and IFN-$\gamma$) [9, 10]. Interestingly, these investigators found that this small molecule inhibitor also potently inhibited TNF-$\alpha$-induced NF-$\kappa$B activation *in vitro* [9]. As a whole, these results suggest that inhibiting SK2 may also represent a good therapeutic approach for IBD [9, 10]. Since SK1 and SK2 are reported to have different biological actions on cellular proliferation and apoptosis [42, 43], it remains to be determined as to which SK isoform represents the best pharmacological target for IBD [9–11]. Nevertheless, targeting the SK pathway (Figure 2) seems to be a rational therapeutic approach for IBD.

## 7. Inhibition of Immunoproteasome Subunits

The constitutive 20S proteasome has a cylindrical structure consisting of three catalytic subunits ($\beta$1, $\beta$2, and $\beta$5). Upon stimulation of cells with proinflammatory cytokines (IFN-$\gamma$ and TNF-$\alpha$), these constitutive subunits are converted to the immunoproteasome subunits $\beta$1i (LMP2), $\beta$2i (LMP10 or MECL-1), and $\beta$5i (LMP7) [38–40]. Functionally, immunoproteasome subunits play a role in MHC class I antigen presentation, as well as NF-$\kappa$B signaling [40, 45–47].

Over the past five years, several research groups (including our own) have suggested a potential role for the immunoproteasome subunits in the pathogenesis of both murine colitis and human IBD [5–8, 33–35]. We showed enhanced expression of the LMP2 (low molecular mass polypeptide 2) subunit in patients with active IBD, particularly in CD patients. Interestingly, LMP2 was also upregulated in areas of the intestine devoid of macroscopic disease [5]. Generally, our results were confirmed by other investigators, who showed significantly enhanced levels of LMP2, LMP7, and LMP10 in CD patients [33–35, 48]. Importantly, in patients with CD, upregulation of the NF-$\kappa$B signal transduction system was observed in the inflamed intestinal mucosa [33].

Using LMP2 knockout mice, we showed that various parameters of DSS-induced colitis (including colonic IL-1$\beta$) were improved compared to WT control mice [6]. Schmidt and colleagues found that parameters of DSS-induced colitis were also attenuated in LMP7-deficient-mice [8]. In these mice, there was diminished activation of the NF-$\kappa$B signal transduction system, resulting in less expansion of Th1 and Th17 T-cells [8]. Basler et al. extended these findings. They showed that mice deficient in any of the immunoproteasome subunits (LMP2, LMP7, and MECL-1) had significant improvements in multiple indices of DSS-induced colitis [7]. Interestingly, significantly reduced levels of Th1 and Th17 cytokines were found in the LMP-deficient mice [7]. As a whole, these data suggest that targeting specific LMP subunits may represent a novel and effective pharmacological strategy for IBD (Figure 2, pathway 2).

From a practical standpoint, targeting specific LMP subunits might best be done by novel chemical inhibitors. Importantly, it has already been shown that treatment with a selective inhibitor of LMP7 (PR-957) strongly suppressed murine DSS-induced colitis [8]. A drug development strategy, using specific LMP proteasome inhibitors (like PR-957), may provide good efficacy in IBD without the side effects of nonselective inhibitors like bortezomib, which also inhibits the constitutive subunits of the proteasome [5–8]. A specific chemical inhibitor of LMP2, designated as UK-101, has also been developed by a research group at the University of Kentucky [49]. This compound should also be tested in animal models of IBD. Finally, selective immunoproteasome inhibitors need to be tested in other colitis models, beyond the testing that has already been completed in the DSS model [8]. Results from these preclinical studies should allow the identification of optimal compound(s) to be progressed into clinical trials for IBD.

## 8. Inhibition of NF-$\kappa$B/STAT3-Signaling Pathways

It has been well documented in the literature that the NF-$\kappa$B pathway, as well as the STAT3 pathway, could be critically involved in the pathogenesis of IBD. Importantly, these papers delineate the roles of these pathways in mediating intestinal inflammation. This literature also points out potential drawbacks of inhibiting NF-$\kappa$B in epithelial cells, as well as blocking STAT3 in epithelial cells and innate immune cells [50–56].

Recently, intriguing information has also been published regarding dual activation of NF-$\kappa\beta$ and STAT3 pathways in pathological conditions such as hepatic inflammation and cancer [12, 57, 58]. It is evident from Figure 2 that NF-$\kappa$B and STAT3 dually control the expression of some target genes (e.g., IL-17), thereby facilitating inflammation [12, 55, 57, 58]. Specifically, it was shown that the canonical NF-$\kappa$B pathway (involving I$\kappa$B-$\alpha$ degradation) and the STAT3 pathway (involving JAK2, PI3K, and AKT1 activation) are both activated by splenic-derived T-cell populations, following dual stimulation with IL-1$\beta$ plus IL-23 (Figure 2) [36, 37]. Sutton and colleagues demonstrated that STAT3

and NF-$\kappa$B pathways mediated IL-17 production from $\gamma\delta$ T-cells [37, 59]. Subsequently, these investigators reported that both $\gamma\delta$ and CD4+ T-cells (via IL-17 production) promoted experimental autoimmune encephalomyelitis (EAE) in mice [59].

It is probable that interactions between the NF-$\kappa$B and STAT3 pathways could also contribute to the pathogenesis of intestinal inflammation/IBD (Figure 2, pathway 3) [12]. Indeed, activation of these pathways was described in conjunction with DSS-induced colitis in mice, as well as in murine TNBS-induced colitis [29, 60]. From a pharmacological development standpoint, there are two key questions that remain to be answered. (1) Are there any small molecule inhibitors that would be good candidates to inhibit interactions between NF-$\kappa$B and STAT3? (2) Would inhibition of these pathways be beneficial?

Indeed, Youn et al. showed that treatment of mice with two plant-derived polyphenols (resveratrol and piceatannol) resulted in the attenuation of DSS-induced colonic inflammation, as well as downregulation of activated NF-$\kappa$B and STAT3 [60]. More recently, we have found that treatment with Vidofludimus attenuated the activation of STAT3 and NF-$\kappa$B pathways, as well as IL-17 production, in murine splenocytes and TNBS-induced colitis [5, 29]. The anticolitis effects that were observed with these chemical compounds are encouraging. However, resveratrol and piceatannol have antioxidant properties, while Vidofludimus can inhibit T-cell proliferation [5, 29, 60]. Therefore, further preclinical colitis studies need to be performed with more specific dual inhibitors of NF-$\kappa$B and STAT3, in order to gauge the clinical potential of this pharmacological approach for IBD. In this regard, a triterpenoid C28 methyl ester derivative (CDDO methyl ester) is an inhibitor of STAT3 (by preventing STAT3 phosphorylation), as well as an inhibitor of NF-$\kappa$B (by inhibiting I$\kappa$B kinase and downstream components of this signal transduction pathway) [61, 62]. Moreover, triterpenoids were effective in preclinical models of pancreatic cancer and cystic fibrosis lung disease [63, 64]. Therefore, CDDO methyl ester, or similar compounds, would be good candidates for testing in preclinical models of IBD.

In summary, all of the potential pharmacological targets (Figures 1 and 2) discussed in this review are upregulated in patients with IBD. Therefore, based on the preponderance of current data, several good opportunities exist for pharmacological inhibition of key components in the IL-23 and IL-17 pathways (Figure 1). These approaches include (1) specific mAb's to TL1A, (2) IL-17 R:Fc fusion proteins, (3) specific antibodies to IL-17F, and (4) small molecule inhibitors like Vidofludimus. Also, other potential opportunities for targeted drug development in IBD include specific chemical inhibitors of SK, specific small molecule inhibitors directed against catalytic subunits of the immunoproteasome, and dual inhibitors of the STAT3 and NF-$\kappa$B signal transduction systems (Figure 2).

In the near future, critically designed preclinical studies are still needed to determine which of these pharmacological approaches will provide drugs with the best efficacy and safety profiles for entrance into clinical trials. Subsequently, well-designed clinical trials are needed to determine the specific pharmacological approaches that will prove to be most successful in patients with IBD.

## References


[1] D. Q. Shih and S. R. Targan, "Insights into IBD pathogenesis," *Current Gastroenterology Reports*, vol. 11, no. 6, pp. 473–480, 2009.

[2] M. Nagahori, Y. Nemoto, and M. Watanabe, "Pathogenesis of inflammatory bowel diseases," *Intest Res*, vol. 8, pp. 9–17, 2010.

[3] J. K. Triantafillidis, E. Merikas, and F. Georgopoulos, "Current and emerging drugs for the treatment of inflammatory bowel disease," *Drug Design, Development and Therapy*, vol. 5, pp. 185–210, 2011.

[4] L. R. Fitzpatrick, L. Deml, C. Hofmann et al., "4SC-101, a novel immunosuppressive drug, inhibits IL-17 and attenuates colitis in two murine models of inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 16, no. 10, pp. 1763–1777, 2010.

[5] L. R. Fitzpatrick, J. S. Small, L. S. Poritz, K. J. McKenna, and W. A. Koltun, "Enhanced intestinal expression of the proteasome subunit low molecular mass polypeptide 2 in patients with inflammatory bowel disease," *Diseases of the Colon and Rectum*, vol. 50, no. 3, pp. 337–350, 2007.

[6] L. R. Fitzpatrick, V. Khare, J. S. Small, and W. A. Koltun, "Dextran sulfate sodium-induced colitis is associated with enhanced low molecular mass polypeptide 2 (LMP2) expression and is attenuated in LMP2 knockout mice," *Digestive Diseases and Sciences*, vol. 51, no. 7, pp. 1269–1276, 2006.

[7] M. Basler, M. Dajee, C. Moll, M. Groettrup, and C. J. Kirk, "Prevention of experimental colitis by a selective inhibitor of the immunoproteasome," *Journal of Immunology*, vol. 185, no. 1, pp. 634–641, 2010.

[8] N. Schmidt, E. Gonzalez, A. Visekruna et al., "Targeting the proteasome: partial inhibition of the proteasome by bortezomib or deletion of the immunosubunit LMP7 attenuates experimental colitis," *Gut*, vol. 59, no. 7, pp. 896–906, 2010.

[9] L. W. Maines, L. R. Fitzpatrick, K. J. French et al., "Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase," *Digestive Diseases and Sciences*, vol. 53, no. 4, pp. 997–1012, 2008.

[10] L. W. Maines, L. R. Fitzpatrick, C. L. Green, Y. Zhuang, and C. D. Smith, "Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease," *Inflammopharmacology*, vol. 18, no. 2, pp. 73–85, 2010.

[11] A. J. Snider, T. Kawamori, S. G. Bradshaw et al., "A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis," *FASEB Journal*, vol. 23, no. 1, pp. 143–152, 2009.

[12] S. Danese and A. Mantovani, "Inflammatory bowel disease and intestinal cancer: a paradigm of the Yin-Yang interplay between inflammation and cancer," *Oncogene*, vol. 29, no. 23, pp. 3313–3323, 2010.

[13] D. Q. Shih, L. Y. Kwan, V. Chavez et al., "Microbial induction of inflammatory bowel disease associated gene TL1A (TNFSF15) in antigen presenting cells," *European Journal of Immunology*, vol. 39, no. 11, pp. 3239–3250, 2009.

[14] D. Q. Shih, K. S. Michelsen, and R. J. Barrett, "Insights into TL1A and IBD pathogenesis," in *Advances in TNF Family Research*, D. Wallach, A. Kovalenko, and M. Feldmann, Eds., pp. 279–288, Springer, New York, NY, USA, 2011.

[15] B. P. Pappu, A. Borodovsky, T. S. Zheng et al., "TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated

autoimmune disease," *Journal of Experimental Medicine*, vol. 205, no. 5, pp. 1049–1062, 2008.

[16] H. Takedatsu, K. S. Michelsen, B. Wei et al., "TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation," *Gastroenterology*, vol. 135, no. 2, pp. 552–e2, 2008.

[17] F. Meylan, Y.-J. Song, I. Fuss et al., "The TNF-family cytokine TL1A drives IL-13-dependent small intestinal inflammation," *Mucosal Immunology*, vol. 4, no. 2, pp. 172–185, 2011.

[18] V. Y. Taraban, T. J. Slebioda, J. E. Willoughby et al., "Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia," *Mucosal Immunology*, vol. 4, no. 2, pp. 186–196, 2011.

[19] G. Bamias, M. Mishina, M. Nyce et al., "Role of TL1A and its receptor DR3 in two models of chronic murine ileitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 22, pp. 8441–8446, 2006.

[20] D. McGovern and F. Powrie, "The IL23 axis plays a key role in the pathogenesis of IBD," *Gut*, vol. 56, no. 10, pp. 1333–1336, 2007.

[21] C. O. Elson, Y. Cong, C. T. Weaver et al., "Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice," *Gastroenterology*, vol. 132, no. 7, pp. 2359–2370, 2007.

[22] M. Elliott, J. Benson, M. Blank et al., "Ustekinumab: lessons learned from targeting interleukin-1223p40 in immune-mediated diseases," *Annals of the New York Academy of Sciences*, vol. 1182, pp. 97–110, 2009.

[23] J. M. Benson, C. W. Sachs, G. Treacy et al., "Therapeutic targeting of the IL-12/23 pathways: generation and characterization of ustekinumab," *Nature Biotechnology*, vol. 29, no. 7, pp. 615–624, 2011.

[24] M. A. Kleinschek, K. Boniface, S. Sadekova et al., "Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation," *Journal of Experimental Medicine*, vol. 206, no. 3, pp. 525–534, 2009.

[25] Z. Zhang, M. Zheng, J. Bindas, P. Schwarzenberger, and J. K. Kolls, "Critical role of IL-17 receptor signaling in acute TNBS-induced colitis," *Inflammatory Bowel Diseases*, vol. 12, no. 5, pp. 382–388, 2006.

[26] A. Strzępa and M. Szczepanik, "IL-17-expressing cells as a potential therapeutic target for treatment of immunological disorders," *Pharmacological Reports*, vol. 63, no. 1, pp. 30–44, 2011.

[27] X. O. Yang, H. C. Seon, H. Park et al., "Regulation of inflammatory responses by IL-17F," *Journal of Experimental Medicine*, vol. 205, no. 5, pp. 1063–1075, 2008.

[28] W. O'Connor, M. Kamanaka, C. J. Booth et al., "A protective function for interleukin 17A in T cell-mediated intestinal inflammation," *Nature Immunology*, vol. 10, no. 6, pp. 603–609, 2009.

[29] L. R. Fitzpatrick, J. S. Small, and A. Ammendola, "Inhibition of IL-17 release by the novel anti-inflammatory drug vidofludimus involves attenuation of STAT3 and NF-kappa B pathways in murine splenocytes and hapten induced colitis," *Gastroenterology*, vol. 140, p. S 837, 2011.

[30] K. R. Herrlinger, M. Diculescu, K. Fellermann et al., "Efficacy, safety, and tolerability of vidofludimus in patients with inflammatory bowel disease: the entrance study," *Gastroenterology*, vol. 140, pp. S588–S589, 2011.

[31] J. F. Colombel, "No response to anti-TNFs; novel agents in the near future," in *Proceedings of the DDW Meeting*, Contemporary Therapeutic Dilemmas in IBD, Chicago, Ill, USA, 2011.

[32] S. Von Vietinghoff and K. Ley, "IL-17A controls IL-17F production and maintains blood neutrophil counts in mice," *Journal of Immunology*, vol. 183, no. 2, pp. 865–873, 2009.

[33] A. Visekruna, T. Joeris, D. Seidel et al., "Proteasome-mediated degradation of IκBα and processing of p105 in Crohn disease and ulcerative colitis," *Journal of Clinical Investigation*, vol. 116, no. 12, pp. 3195–3203, 2006.

[34] A. Visekruna, T. Joeris, N. Schmidt et al., "Comparative expression analysis and characterization of 20S proteasomes in human intestinal tissues: the proteasome pattern as diagnostic tool for IBD patients," *Inflammatory Bowel Diseases*, vol. 15, no. 4, pp. 526–533, 2009.

[35] A. Visekruna, N. Slavova, S. Dullat et al., "Expression of catalytic proteasome subunits in the gut of patients with Crohn's disease," *International Journal of Colorectal Disease*, vol. 24, no. 10, pp. 1133–1139, 2009.

[36] M. L. Cho, J. W. Kang, Y. M. Moon et al., "STAT3 and NF-κB signal pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice," *Journal of Immunology*, vol. 176, no. 9, pp. 5652–5661, 2006.

[37] C. Sutton, C. Brereton, B. Keogh, K. H. G. Mills, and E. C. Lavelle, "A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis," *Journal of Experimental Medicine*, vol. 203, no. 7, pp. 1685–1691, 2006.

[38] S. Scheffler, U. Kuckelkorn, K. Egerer et al., "Autoimmune reactivity against the 20S-proteasome includes immunosubunits LMP2 ($\beta$1i), MECL1 ($\beta$2i) and LMP7 ($\beta$5i)," *Rheumatology*, vol. 47, no. 5, pp. 622–626, 2008.

[39] M. Aki, N. Shimbara, M. Takashina et al., "Interferon-$\gamma$ induces different subunit organizations and functional diversity of proteasomes," *Journal of Biochemistry*, vol. 115, no. 2, pp. 257–269, 1994.

[40] T. Hayashi and D. Faustman, "Essential role of human leukocyte antigen-encoded proteasome subunits in NF-κB activation and prevention of tumor necrosis factor-α-induced apoptosis," *Journal of Biological Chemistry*, vol. 275, no. 7, pp. 5238–5247, 2000.

[41] M. Maceyka, H. Sankala, N. C. Hait et al., "SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism," *Journal of Biological Chemistry*, vol. 280, no. 44, pp. 37118–37129, 2005.

[42] J. S. Karliner, "Sphingosine kinase regulation and cardioprotection," *Cardiovascular Research*, vol. 82, no. 2, pp. 184–192, 2009.

[43] P. Xia, J. R. Gamble, K. A. Rye et al., "Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 24, pp. 14196–14201, 1998.

[44] L. Zhi, B. P. Leung, and A. J. Melendez, "Sphingosine kinase 1 regulates pro-inflammatory responses triggered by TNFα in primary human monocytes," *Journal of Cellular Physiology*, vol. 208, no. 1, pp. 109–115, 2006.

[45] J. D. Mountz, "Significance of increased circulating proteasome in autoimmune disease," *Journal of Rheumatology*, vol. 29, no. 10, pp. 2027–2030, 2002.

[46] M. Groettrup and G. Schmidtke, "Selective proteasome inhibitors: modulators of antigen presentation?" *Drug Discovery Today*, vol. 4, no. 2, pp. 63–71, 1999.

[47] M. Groettrup, S. Khan, K. Schwarz, and G. Schmidtke, "Interferon-$\gamma$ inducible exchanges of 20S proteasome active site subunits: why?" *Biochimie*, vol. 83, no. 3-4, pp. 367–372, 2001.

[48] M. Coëffier, R. Gloro, N. Boukhettala et al., "Increased proteasome-mediated degradation of occludin in irritable bowel syndrome," *American Journal of Gastroenterology*, vol. 105, no. 5, pp. 1181–1188, 2010.

[49] Y. K. Ho, P. Bargagna-Mohan, M. Wehenkel, R. Mohan, and K. B. Kim, "LMP2-Specific inhibitors: chemical genetic tools for proteasome biology," *Chemistry and Biology*, vol. 14, no. 4, pp. 419–430, 2007.

[50] I. Atreya, R. Atreya, and M. F. Neurath, "NF-$\kappa$B in inflammatory bowel disease," *Journal of Internal Medicine*, vol. 263, no. 6, pp. 591–596, 2008.

[51] C. Jobin, "Nf-kappa B signaling cascade and IBD: turn it down?" *Inflammatory Bowel Diseases*, vol. 14, supplement 2, pp. S108–S109, 2008.

[52] K. Sugimoto, "Role of STAT3 in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 14, no. 33, pp. 5110–5114, 2008.

[53] K. Mitsuyama, S. Matsumoto, J. Masuda et al., "Therapeutic strategies for targeting the IL-6/STAT3 cytokine signaling pathway in inflammatory bowel disease," *Anticancer Research*, vol. 27, no. 6 A, pp. 3749–3756, 2007.

[54] J. Wei and J. Feng, "Signaling pathways associated with inflammatory bowel disease," *Recent Patents on Inflammation and Allergy Drug Discovery*, vol. 4, no. 2, pp. 105–117, 2010.

[55] J. Kurtovic and I. Segal, "Recent advances in biological therapy for inflammatory bowel disease," *Tropical Gastroenterology*, vol. 25, no. 1, pp. 9–14, 2004.

[56] C. Neufert, G. Pickert, Y. Zheng et al., "Activation of epithelial STAT3 regulates intestinal homeostasis," *Cell Cycle*, vol. 9, no. 4, pp. 652–655, 2010.

[57] S. I. Grivennikov and M. Karin, "Dangerous liaisons: STAT3 and NF-$\kappa$B collaboration and crosstalk in cancer," *Cytokine and Growth Factor Reviews*, vol. 21, no. 1, pp. 11–19, 2010.

[58] G. He and M. Karin, "NF-$\kappa$B and STAT3-key players in liver inflammation and cancer," *Cell Research*, vol. 21, no. 1, pp. 159–168, 2011.

[59] C. E. Sutton, S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. G. Mills, "Interleukin-1 and IL-23 induce innate IL-17 production from $\gamma\delta$ T cells, amplifying Th17 responses and autoimmunity," *Immunity*, vol. 31, no. 2, pp. 331–341, 2009.

[60] J. Youn, J. S. Lee, H. K. Na, J. K. Kundu, and Y. J. Surh, "Resveratrol and piceatannol inhibit iNOS expression and NF-kappaB activation in dextran sulfate sodium-induced mouse colitis," *Nutrition and Cancer*, vol. 61, no. 6, pp. 847–854, 2009.

[61] R. Ahmad, D. Raina, C. Meyer, and D. Kufe, "Triterpenoid CDDO-methyl ester inhibits the Janus-activated kinase-1 (JAK1) → signal transducer and activator of transcription-3 (STAT3) pathway by direct inhibition of JAK1 and STAT3," *Cancer Research*, vol. 68, no. 8, pp. 2920–2926, 2008.

[62] S. Shishodia, G. Sethi, M. Konopleva, M. Andreeff, and B. B. Aggarwal, "A synthetic triterpenoid, CDDO-Me, inhibits I$\kappa$B$\alpha$ kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor $\kappa$B-regulated gene products in human leukemic cells," *Clinical Cancer Research*, vol. 12, no. 6, pp. 1828–1838, 2006.

[63] K. T. Liby, D. B. Royce, R. Risingsong et al., "Synthetic triterpenoids prolong survival in a transgenic mouse model of pancreatic cancer," *Cancer Prevention Research*, vol. 3, no. 11, pp. 1427–1434, 2011.

[64] D. P. Nichols, A. G. Ziady, S. L. Shank, J. F. Eastman, and P. B. Davis, "The triterpenoid CDDO limits inflammation in preclinical models of cystic fibrosis lung disease," *American Journal of Physiology*, vol. 297, no. 5, pp. L828–L836, 2009.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 493717, 19 pages
doi:10.1155/2012/493717

*Review Article*

# Carbohydrate Elimination or Adaptation Diet for Symptoms of Intestinal Discomfort in IBD: Rationales for "Gibsons' Conundrum"

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Received 15 September 2011; Revised 13 November 2011; Accepted 14 November 2011

Academic Editor: David Sachar



Therapeutic use of carbohydrates in inflammatory bowel diseases (IBDs) is discussed from two theoretical, apparent diametrically opposite perspectives: regular ingestion of prebiotics or withdrawal of virtually all carbohydrate components. Pathogenesis of IBD is discussed connecting microbial flora, host immunity, and genetic interactions. The best studied genetic example, NOD2 in Crohn's disease, is highlighted as a model which encompasses these interactions and has been shown to depend on butyrate for normal function. The role of these opposing concepts in management of irritable bowel syndrome (IBS) is contrasted with what is known in IBD. The conclusion reached is that, while both approaches may alleviate symptoms in both IBS and IBD, there is insufficient data yet to determine whether both approaches lead to equivalent bacterial effects in mollifying the immune system. This is particularly relevant in IBD. As such, caution is urged to use long-term carbohydrate withdrawal in IBD in remission to control IBS-like symptoms.

## 1. Introduction

A conundrum is defined by the American Heritage Dictionary of the English language [1] as "a riddle, especially one whose answer makes a play on words or as a puzzling question or problem." In 1995, Gibson and Roberfroid published their treatise on the potential benefits of maldigested carbohydrates on host health through manipulation of microflora [2]. The concept of prebiotics (nondigestible, highly fermentable, dietary substances that exhibit beneficial functions in the host by facilitating the growth and metabolic activity of either one or a selective number of health-promoting colonic species) coincided with the emergence of potential human benefits found in probiotics (live bacteria bypassing the acid environment of the stomach and conferring health benefits to the host. A combination of pre- and probiotics is referred to as a synbiotic). A deluge of basic and clinical studies ensued as well, on the effects of prebiotics on an array of diseases. In particular, Crohn's disease (CD) and idiopathic ulcerative colitis (UC) (the two clinical subtypes of IBD) were targeted to capitalize on the potential therapeutic effects of either pro- or prebiotics [3–5]. While CD and idiopathic UC both share somewhat similar epidemiology and are thought to have originated from common genetic and environmental etiogenesis, they are in fact considered as two different entities. CD is unrestricted to any part of the gastrointestinal tract, in which the terminal ileum with or without the proximal colon remains the most common site affected. In UC, pathology tends to begin in the distal rectum and then it may proceed to involve the rest of the colon in a uniform fashion.

Similarly a benign but lifestyle-altering condition of irritable bowel syndrome (IBS—a chronic functional bowel disorder encompassed by frequent recurrences of abdominal pain is associated with altered bowel movements: diarrhea, constipation, or alternating form) also fell into the category potentially ameliorated by probiotics and perhaps prebiotics. In both of these conditions, however, it was postulated

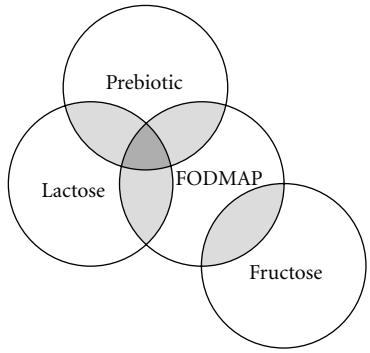


Figure 1: This Venn diagram shows the relationship between FODMAP, comprises of fructose, oligosaccharides, disaccharides, monosaccharides, and polyols. The central diet includes the majority of carbohydrates which are hypothesized to be malfermented by lower intestinal bacteria and therefore leading to excess production of gas and short-chain fatty acids with induction of symptoms. Thus, FODMAP includes all prebiotics in which lactose is included also as a restricted probiotic in lactose maldigesters. It is the hypothetical benefits of either withdrawal from diet or adapting to the prebiotic components of this diet that potentially forms a scientific conundrum in application.

that bacterial interactions, abnormal fermentation, and host handling of fermentative products as well as an immune response rather contributed to aggravation of symptoms [6, 7]. In 2005, Gibson and Shepherd hypothesized such mechanisms in causation of gastrointestinal symptoms in these disorders and suggested that carbohydrates be withdrawn from diets of symptomatic IBS or IBD patients. This FODMAP diet suggests the withdrawal of fermentable oligo-, di-, monosaccharides, and polyols from the diet [8]. As such, the FODMAP diet includes lactose and most other prebiotics (refer to Figure 1 and Table 1). Some of these recommendations, of careful carbohydrate selection for diet in patients with IBD, were also suggested earlier in a book by Gottschall [9]. There was less emphasis on small molecules except for sweeteners and more on large complex carbohydrates.

The presentation of these two hypotheses, then, formulates a conundrum. In the first instance, carbohydrates bypassing absorption in the small intestine can specifically manipulate metabolism and benefit the commensal bacteria, which in turn help reduce inflammation. It is important to note that there are specific recognized prebiotics, but all carbohydrates interact with the microbiome. In the second scenario, a wide array of carbohydrates, including prebiotics, are withdrawn. Are the end results equivalent, that is, has a stimulated immune system been placated? A similar paradigm applies to use of probiotics [10] or antibiotics which have received some success both in IBD [11] and IBS [12–14] as well as to other dietetic interventions. These include enteral/polymeric diets and nonspecific exclusion diets that have previously been implicated for their therapeutic roles which are beyond the scope of this review [15].

Herein we will focus on discussing the rationales behind the usage or nonusage of FODMAP diet in IBD. Effects in IBS will be discussed overall, but the concept will be discussed in detail as it might apply to IBD based on current concepts of pathogenesis. The objectives are to review carbohydrate pathogenic interactions with intestinal immunity and to conceive an effective intervention that convenes the apparent hypothetical contradictions inherent in the two approaches to carbohydrate use.

## 2. Microbial Diversity in the Gut

*2.1. Normal Development.* The gastrointestinal microbiota (or microflora) differs among individuals and its dominant bacterial phylotypes are acquired from the moment of birth. Although intestinal microbial composition will remain fairly constant from early infancy throughout adulthood once bacterial colonization is established [16], these microorganisms respond adaptively to better accommodate and protect at an individual level. As such, fecal samples adequately reflect the colonic bacterial environment and indicate the individual's intestinal status in response to developmental changes, environmental factors, antibiotic usage, or illness. The recent advancement in molecular profiling methods, such as high-throughput sequencing of microbial 16S ribosomal RNA genes [17] and metagenomics [18], provides a comprehensive insight into the 100 trillion bacteria that currently comprise the microbiota in the distal gastrointestinal tract alone. Representing the two predominant phylotypes found in mucosal and luminal microbiota are Gram-positive Firmicutes (species including Clostridia and Lactobacillaceae) and Gram-negative Bacteroidetes (species including Bacteroides), all of which are obligatory anaerobic bacteria.

*2.2. Functions of the Microbiome.* Sharing a symbiotic relationship with a dynamic bacterial community also means acquiring a diverse metabolic profile essential for intestinal development [19]. The resident microflora promotes the differentiation and proliferation of enteric epithelial cells by harvesting essential minerals (e.g., iron, calcium, and magnesium) as well as mediating the synthesis of vitamins (e.g., cobalamin, vitamin K, biotin, pyridoxal phosphate, and tetrahydrofolate). In addition, the bacterial genome (also known as the microbiome) encodes a large repertoire of saccharolytic enzymes, including glycoside hydrolases and polysaccharide lyases, needed to further metabolize nondigestible carbohydrates such as plant polysaccharides (dietary fibers), oligosaccharides, lactose (especially in lactose maldigesters), and sugar alcohols in the proximal colon through a process called saccharolytic fermentation [18]. This colonic fermentation of macronutrients yields various end products like gaseous compounds (e.g., hydrogen gas, methane, and carbon dioxide) and short-chain fatty acids (SCFAs), with the latter being mostly comprised of acetate, propionate, and butyrate. These are utilized as the primary energy source for the colonic mucosa. Colonic concentration of SCFA substrates is determined not only by the consumption of dietary fiber but also by the bacterial species present in the microbiota. For example, the two prominent bacterial

Table 1: List of poorly digested carbohydrates comprised of FODMAP and select prebiotics (∗), as well as their respective sources. This is not a complete list, and other complex carbohydrates which have effects on bacteria are also included in FODMAP.

| Molecular form | Common sources |
|---|---|
| Inulin (∗) | Onions, leeks, chicory, artichoke, wheat, banana |
| Oligofructose (∗) | Hydrolysis product of inulin |
| Short-chain fructo-oligosaccharide (∗) | Hydrolysis product of inulin |
| Trans galacto-oligosaccharides (∗) | Manufactured from lactose |
| Lactulose (∗) | Manufactured from lactose |
| Fructo-oligosaccharides (∗) | May be present in breast milk, formed from lactose |
| Isomalto-oligosaccharides (∗) | Present in foods, potential prebiotic |
| Lactose (∗) | Present in dairy products made from animal sources, prebiotic mostly in lactose maldigesters |
| Polyols | Sugar alcohols (sorbitol, mannitol, xylitol, maltitol, and isomalt), cauliflower, avocado, mushrooms |

phylotypes each differs in the types of SCFAs produced, with Firmicutes selectively producing butyrate and Bacteroidetes controlling the levels of acetate and propionate production [20, 21]. Small carbohydrates like lactose may also lead to production of butyrate through the stimulation of second tier bacteria (butyrogens) by initial breakdown products [22, 23]. The presence of these organic acids helps induce an acidic environment unfavourable for the proliferation of strict anaerobic species [24, 25]. Once carbohydrates are no longer available for fermentation, bacteria will proceed to proteolytic fermentation (less favourable) in the distal colon where proteins derived from diet, endogenous cellular proteins, and bacterial cells are catalyzed to toxic, carcinogenic metabolites (e.g., bacteriocins, ammonia, indoles, and phenols) [26]. These substances inhibit the growth or kill potentially pathogenic constituents. Another way the microbiota maintains resistance against colonization by pathogenic organisms is to compete for nutrients and attachment sites to the mucosal surface in the colon [27, 28]. Minor perturbations in the intricate microbial diversity can have significant impact on the gut homeostatic balance [29–32]. These changes have been implicated to predispose or contribute to conditions such as sepsis, IBS symptoms [6, 33], and even obesity in some populations [34, 35].

## 3. The Interaction of the Microbiome with Intestinal Mucosal Immunity

*3.1. Mucosal Immune System.* Intestinal mucosal immunity is associated with the integrity of the intracellular junctions in the gut epithelium constituting what is called a physical barrier. The mucosal integrity is further strengthened by what is called a chemical barrier thanks to a specialized group of differentiated epithelial cells residing in the paracellular space. Goblet cells, for instance, are responsible for secreting an overlying glycocalyx layer composed of mucin glycoproteins [36]; production of defensins, immunoglobulins, and other substances by enterocytes, lymphocytes, and Paneth cells (the last being generally restricted to the crypts of Lieberkuhn in the distal small bowel) can also be found within this mucus layer [37, 38]. This dual barrier provides enhanced protection against unwarranted entry of luminal contents (including self- and non-self-antigens) into the systemic immune system, but this is also where innate immune recognition takes place. Many of the cells in this mucosal barrier respond to pathogens by expressing two functionally important subsets of pattern-recognition receptors (PRRs)—extracellular Toll-like receptors (TLRs) and intracellular nuclear oligomerization domain-(NOD-) like receptors. These assist in the detection of pathogen-associated molecular patterns (PAMPs) through the leucine-rich repetitive (LRR) domain. Lipopolysaccharides (LPSs) and peptidoglycan (PGN) components (i.e., muramyl dipeptide) of bacterial cell wall are two examples of PAMPs. Each subset can either individually or convergently activate nuclear factor $\kappa$B (NF-$\kappa$B) effector in the defense against foreign pathogens by producing inflammatory cytokines (e.g., TNF-$\alpha$ and IL-1$\beta$) and antimicrobial peptides [39]. Chronic stimulation of PRRs by PGN can also produce inhibitory cytokines (e.g., TGF-$\beta$ and IL-10) via the NOD2-dependent pathways to minimize excessive tissue injury induced by intestinal antigen-presenting cells [40]. Intestinal mucosal immunity is reinforced further by continuous interaction between epithelial cells and adaptive immune cells, including effector T-helper cells (Th1, Th2, and Th17), regulatory T cells (Foxp3+ Treg), and other immune cells (i.e., dendritic cell, macrophage, and natural killer cell) at the follicle-associated epithelium junction overlying the gut-associated lymphoid tissue [41].

Central to the discussion in conferring protection to the host is the influences of microbiota community on the normal development and homeostasis of mucosal immunity [52–54]. The symbiotic nature of the host-microbiota relationship is fundamental to the shaping of immunological function, balance, and tolerance in the gut. Paradoxically, the key for preserving such symbiotic coexistence in return depends on the robustness of the intestinal immune network, particularly in its ability to differentiate between symbiotic and pathogenic colonization. The maintenance of gut homeostatic balance, therefore, depends on the cooperation between mucosal immunity and microbial community, that is, if the right microbiota composition is present. Alteration to the microbial ecology, commonly referred to as dysbiosis, can distort intestinal immune responses by shifting the equilibrium between pro- and anti-inflammatory T-helper cells differentiation, as characterized by IBD pathogenesis (Box 1) [55–57].

Classically, IBD (especially in CD) is associated with a hyperactive innate immune response producing unrestrained levels of proinflammatory cytokines and chemokines (e.g., IL-12, IFN-$\gamma$, and TNF-$\alpha$), resulting in a marked expansion of lamina propria. This propagates further inflammation by recruiting T-helper 1 ($CD4^+$ Th1) cells. Alternatively, the opposite scenario can occur in which resident tissue macrophages fail in their attempt to initiate an innate immune response against foreign antigens and are defective in the secretion of proinflammatory cytokines [42, 43]. Reduced concentrations of these mediators mean neutrophil recruitment cannot be adequately enforced at the lamina propria, resulting in impaired clearance of antigenic contents [44]. The following overcompensatory immune responses lead to either a polarization toward an atypical humoral phenotype driven by T-helper 2 ($CD4^+$ Th2) cells along with mediators such as IL-4 and IL-13 (especially in UC [45]) or recruitment of $CD4^+$ Th1 cells [36]. The amplification of inflammatory response as an attempt to remove foreign material only incites further epithelial injury which coincides with a decreased production of defensins [46, 47]. It is quite possible that both paradigms may be true given the genetic heterogeneity among IBD populations. A newly discovered subset of inflammatory T cells, known as T-helper 17 (Th17) cells, produces the proinflammatory cytokine IL-17 and requires IL-23 for proper maintenance and function. Indirectly, Th17 cells relate CD and UC etiologies due to IL-23 sharing similar subunits with another major cytokine found in the Th1 phenotype, namely, IL-12 [48, 49]. Also, responsiveness to anti-TNF-$\alpha$ treatment suggests common pathogenic pathways are shared by both IBD subtypes [50, 51].

Box 1: IBD pathogenesis.

*3.2. Concept of Dysbiosis and IBD.* Analyses in the gastrointestinal microbial populations showed significant differences between healthy individuals and patients with IBD, an indication that dysbiosis may be a contributing factor to IBD [58, 59]. Specifically, an increased propensity of obligatory aerobic bacteria is seen displacing the anaerobic species, with Bifidobacteria (in CD) [60] and Lactobacilli (in UC) [20] both being deficient in the microbiota. Reduced diversity of mucosa-associated phyla Firmicutes and Bacteroidetes is commonly observed as well in IBD controls [59, 61]. Depletion of *Faecalibacterium prausnitzii* is related to an activated immune response, which specifically suppresses and eradicates selective groups of bacteria resulting in an imbalance of intestinal flora [62]. This is relevant due to *F. prausnitzii* belonging to the genus Firmicutes in the Clostridia 14 cluster, which in fact is an important butyragenic-stimulated bacterium capable of exerting anti-inflammatory effects [63].

Swidsinski et al. has shown that a person afflicted with IBD displays an intestinal mucosa heavily populated with adherent organisms which are virtually nonexistent in a healthy individual [64]. For instance, adherent-invasive E. coli are isolated and found to adhere to the brush border of primary ileal enterocytes of CD patients but none in healthy controls [65–67]. Most recently, however, Willing et al. demonstrated that specific bacterial changes were associated with different anatomical sites in CD but UC patients in remission shared a similar microflora as to healthy controls [68]. This correlates with the data gathered from a comparative microbiota analysis of mice where they found closely related phylotypes displayed higher abundances (cooccurrence) and are conducive to intestinal colonization irrespective of the microbial origin (external or internal) [69]. Highly abundant subsets of commensal microorganisms, such as *Helicobacter*, *Clostridium*, and *Enterococcus* species, are hence more susceptible to transform the symbiotic nature of the host-microbiota relationship into a pathogenic one under certain environmental conditions [54]. Mucosal antibodies recovered from IBD subjects are found to be directed against intestinal commensal bacteria, as such, they may be more responsive to antibiotic treatment and faecal diversion than non-IBD controls [70, 71].

## 4. The Relationship of Bacterial Metabolites of Carbohydrates and Mucosal Immunity

*4.1. Immunoregulatory Functions of Short-Chain Fatty Acids.* Given that environmental-induced changes can alter the intestinal microbiota, leading to dysregulatory inflammatory responses, increasing evidence indicates that microbial fermentative by-products (e.g., acetate, propionate, and butyrate) demonstrate anti-inflammatory properties that may be clinically relevant to the treatment of IBD [14, 77, 90–93]. One study attributed the interaction between acetate and the chemoattractant receptor, G-protein-coupled receptor 43 (GPR43; also referred to as FFAR2) [94], critical in the regulation as well as the resolution of inflammatory responses [95]. By analyzing the transcription profiles of cellular receptor genes found in human leukocytes, the investigators had identified high degree of GPR43 expression in neutrophils and eosinophils; its expression was also closely governed by Toll-like receptors (TLR2 and TLR4), formyl peptide receptors (FPR1 and FPR2), and C5aR suggesting that GPR43 is important for innate immune and chemoattractant-induced responses. To examine the anti-inflammatory protection conferred by the acetate-GPR43 signalling pathway, they induced acute colitis by adding dextran sulphate sodium (DSS) to the drinking water of GPR43-deficient ($Gpr43^{-/-}$) and wild-type mice for one

Table 2: Some immunoregulatory functions of butyrate.

| | |
|---|---|
| (i) Increases choline acetyltransferase immunoreactive (ChAT-IR) enteric neurons in vivo and in vitro<br>(ii) Increases cholinergic-mediated colonic motility and contractile response ex vivo | [72] |
| (i) Modulates oxidative stress in healthy colonic mucosa<br>(ii) Promotes glutathione (GSH) and lower uric acid concentrations compared | [73] |
| (i) Promotes the differential expression of 500 genes in human colonic mucosa<br>(ii) Increases gene expression of transcriptional regulation pathways: fatty acid oxidation, electron transport chain, and oxidative stress<br>(iii) Increases gene expression related to epithelial integrity and apoptosis | [74] |
| (i) Influences colonic function, mainly by histone deacetylase inhibition | [75, 76] |
| (i) Reduces inflammatory responses in vitro, mainly by inhibition of NF-$\kappa$B activation | [77] |
| (i) Mediates NOD2-dependent mucosal immune responses against PGN | [78] |
| (i) Modulates an intracellular JAK/STAT1 signaling cascade which inhibits NO production | [79] |
| (i) Enhances upregulation/detection of PRRs on intestinal epithelial cells | [80–83] |
| (i) Anticarcinogenic/angiogenic by modulating the activity of several key regulators involved in apoptosis and cell differentiation | [84–86] |
| (i) Enhances colonic defense barrier | [87–89] |

week. Compared to the wild-type, *Gpr43*$^{-/-}$ mice exhibited exacerbated inflammatory response based on histological analysis, daily activity index (DAI; a combined measure of weight loss, rectal bleeding, and stool consistency), and increased levels of myeloperoxidase activity (MPO; inflammatory mediator) in the colon. A significant improvement to those inflammatory parameters soon followed after 200 mM acetate was introduced in their drinking water in a GPR43-dependent manner (*Gpr43*$^{-/-}$ mice lacked the receptor to respond to acetate but not in wild-type ones). Similar development of unresolved inflammation occurred in other mice models such as DSS-induced colitis in germ-free wild-type, K/BxN serum-induced model of inflammatory arthritis and ovalbumin-induced model of allergic airway inflammation. Host protection against enteropathogen *Escherichia coli* (0157:H7) infection was recently linked to acetate production by Bifidobacteria [96]. They proposed that acetate prevented the pathogen from entering the systemic circulation by enhancing mucosal barrier defense.

Although acetate and propionate have long been shown to exert immunologic modification [14], it is butyrate which generates the majority of interest in research. The immunoregulatory activities exerted by butyrate are listed in Table 2. Butyrate is able to regulate multiple gene expressions in the colonic epithelial cells [74, 75]. Inhibition of histone deacetylase by butyrate has been identified to orchestrate a series of downstream effectors responsible for its attributive anti-inflammatory profile [76, 97]. Most notably is the direct suppression of the NF-$\kappa$B transcription factor via histone acetylation, which in turn alters the transcriptional patterns of many genes encoding cytokines, chemokines, adhesion molecules, and other proinflammatory mediators [77, 98–101].

Other anti-inflammatory properties of butyrate highlighted as possible therapeutic targets in IBD include its ability to modulate an intracellular JAK/STAT1 signalling cascade which reduces NO production in macrophages and in intestinal myofibroblasts [79]; enhance the upregulation/detection of PRRs on intestinal epithelial cells (e.g., TLR1, TLR4, TLR6, peroxisome proliferator-activated receptor-$\gamma$ (PPAR$\gamma$)) [80–83], hence facilitating the migration of neutrophil [102]; mitigate the extent of DNA damage in colonocytes induced by neutrophilic oxidizing species during carcinogenesis [103, 104]; potentiate the expression of heat shock proteins, especially HSP70 and HSP25, in enterocyte-like Caco-2 cells and DSS-induced colitis which further enhances cellular protection during an inflammatory response [105, 106].

In some cultured cell lines, butyrate improved the status of intestinal defense mechanisms commonly impaired in IBD by restoring mucosal barrier integrity and promoting epithelial migration in a dose-dependent manner [87–89]. Specifically, its administration has been demonstrated to stimulate MUC2 mucin gene expression in which its protein product is often altered in IBD [107–109]. An increased mucin secretion has also been reported in the isolated vascularly perfused rat colon [110]. Butyrate was also demonstrated to modulate the expression of antimicrobial peptide, cathelicidin (LL-37), in isolated colon epithelial cell lines [111]. A reinforced mucus layer and epithelial tight junctions mean decreasing mucosal permeability, making foreign substances impossible to pass through the defense barrier.

To date potential therapeutic effects of butyrate have been limited to UC. Interestingly, in vivo studies have shown that butyrate oxidation in the colon mucosa of patients with quiescent UC remain normal, whereas those with an actively inflamed mucosa do not [112]. It was reported that TNF-$\alpha$, an inflammatory mediator, may be responsible for the reduced colonic uptake of butyrate [113]. The deprivation of butyrate or any other SCFAs, in conjunction with the toxic metabolites derived from proteolytic fermentation when saccharolytic fermentation is not possible, has long been proposed for the pathogenesis of gastrointestinal disorders

(or even cancer) identified to originate in the distal colon [114]. Similar proposal concerning their therapeutic role in the regulation of inflammatory immune responses and the defense of mucosal immunity with respect to cellular functions in the colon is also made [26, 115–117]. The therapeutic effects of either butyrate alone or combination of SCFAs on patients with moderate-to-active colonic inflammation were confirmed. Many of the UC patients showed responsiveness toward rectal enema treatment of butyrate (amid methodological and procedural differences), whereby symptomatic improvement was reported afterward and coincided with a reduction in the inflammatory parameters [93, 118, 119]. Despite the fact that clinical data have not established an efficacious dietary quantity/frequency of butyrate [120–122], current in vitro and ex vivo studies do implicate a regulatory role in intestinal mucosal immunity.

## 5. Genes and IBD

Disease expression observed in individuals with IBD are a result of genetic predisposition to mounting an inappropriate inflammatory response toward commensal microflora (i.e., anergy is breached) [123, 124]. Some view immunodeficiency phenotype as the principle drive behind IBD pathogenesis [125, 126], but external variables including degree of bacterial load, malnutrition, surgery, and/or use of immunosuppressant therapy must be present in order to facilitate the disruption of the mucus layer and/or epithelial tight junctions. As a result, rendering the submucosal compartments to become increasingly susceptible to bacterial exposure, penetration, and adherence [36, 127–130]. Most importantly, these variables predispose to abnormal interactions with the microbiota. Whether observed dysbiosis, particularly in CD, is a result of the host reaction and/or therapy or a precursor to disease development is unclear yet.

Early progress was centered on characterizing genetic variations in association with IBD susceptibility as supported by familial aggregation studies and population-based cohort surveys. It was suggested that geographic location, ethnic background, socioeconomic class, and positive familial IBD history (e.g., first-degree relatives and monozygotic twin) are all variables dictating the risk in an individual for developing IBD [131, 132]. Out of the 71 CD candidate genes and 47 for UC that have been identified to date [133], only about 30 of them are clearly delineated [50]. Genetic studies are providing more concrete evidence for earlier epidemiological studies, but at the same time pose additional questions that further highlight the complex etiologies associated with IBD. Despite some similar phenotypic traits, IBD subtypes do not share all susceptibility loci. Another key finding reveals that allelic variants to date confer to only a small fraction of disease heritability in the IBD populations. This suggests that as yet unidentified genes or other environmental factors are attributable to IBD pathophysiological development. Indeed in CD genetic predisposition to bacterial infections is generally not enough to bring forth the clinical symptoms. A number of additional environmental factors have now been delineated, and these include smoking (promotes CD and protects against UC) [134], appendectomy (may promote CD and protects against UC) [135], nonsteroidal anti-inflammatory drugs (promote CD) [134], bacterial or viral infections (disrupt mucosal permeability of the intestine) [134], and early exposure to antibiotics (promote CD) [136].

*5.1. Mechanistic Model of Genetic, Nutrient, Microbial Interaction: Function of NOD2.* The first and most consistent mutations associated with increased susceptibility of CD (but not UC) are in the nucleotide-binding oligomerization domain containing 2 (NOD2) gene located on chromosome 16q12. Formerly it was known as caspase activated recruitment domain protein 15 (CARD15) gene [39]. Considerable research has revealed a complex of interactive components necessary for the normal function of disposing the host of bacterial invaders. This section reviews the components, genetic and dietary, needed for such function. It is used primarily here as an example of the mechanistic interactive effects outlined above and how dysfunctions in different components could lead to disease.

Nod2 protein (product of the NOD2 gene) belongs to the family of PRRs. Upon recognition of bacterial-associated PGN patterns, it mediates the activation of two pathways—NF-$\kappa$B and mitogen-activated protein (MAP) kinase. This intracellular receptor located predominantly in Paneth and other cells situated in the distal ileum plays a key part in the innate immune defense by eliminating intracellular bacteria or bacterial debris [137]. Its genetic mutations confer susceptibility in CD mice models [138]. Three major NOD2 mutations associated with the LRR domain have been confirmed: two missense SNPs (Arg702Trp and Gly908Arg) and one frameshift variant (Leu1007fsinsC), respectively [139–141]. All three mutations share similar restricted activation of the NF-$\kappa$B pathway in response to LPS and PGN treatments [140, 142, 143]. Despite the prevalence of NOD2 mutations present among the Caucasian populations (approximately 30% of patients of European ancestry have at least one of the three polymorphisms), the genetic penetrance corresponds to less than 10% of CD manifestation found in the carriers [144, 145].

*5.2. Genetic Components for Normal NOD2 Function.* A number of genes interact to promote normal NOD2 function. These include genes controlling Toll-like receptors, autophagy genes (ATG16L1 and IRGM), and most recently, products of Transducin-like enhancer of split 1 (TLE1) also demonstrate major effects. Even though loss of function/regulation in NOD2 may not compromise NF-$\kappa$B signalling completely, an imbalance of immune activity among mucosal cells is often the case due to oversecretion of proinflammatory cytokines as an attempt to dispose bacterial components [146]. A recent paper reported that TNF receptor 4 (TRAF4) is responsible for downregulating the activation of NF-$\kappa$B, hence limiting the innate response. This indicates that mutations in this downregulator may be key in correcting the acute innate response similar to how bacterial inoculation could do for NOD2 polymorphisms [147].

Autophagy is a highly conserved cellular process recognized for its role during starvation and in intracellular pathogen clearance. In the former, intracellular components are degraded indiscriminately to ensure cell viability. In the latter case, the process involves the precise rearrangement of intracellular constituents (e.g., bacteria, mitochondria, intracellular membranes, and proteins) to form a macroautophagy structure in order to isolate the foreign pathogen for digestion. It is then sequestered in a double-membrane cytosolic vacuole called an autophagosome which later fuses with lysosomes for further processing [148, 149]. Genome-wide association (GWA) studies have identified two sequence variants involved in the autophagy pathway, ATG16L1 and IRGM1, which confer to the genetic susceptibility of CD [150–153]. Although the functional consequences as to how ATG16L1 and IRGM1 mutations contribute to the pathogenesis of CD are not fully understood, accumulating human genetic data suggest that the location of ATG16L1 risk allele on chromosome 2q37 might be linked to autophagy mutations found in macrophage and Paneth cell.

Most recently a number of proteins have been identified in vitro to interact with NOD2 [154]. Using a yeast 2-hybrid screen some have been connected to a gene TLE1 which affects mucin biosynthesis and apoptosis. These epistatic interactions are putatively regulatory, and mutations in one of the alleles of TLE1 appear to be necessary for CD risk in the presence of classical NOD2 mutations. This allele may also increase the risk for UC which is independent of NOD2 mutations.

*5.3. Nutrient Components for Normal NOD2 Function.* In addition to the genetically mediated controls outlined for NOD2, two environmental variables have been shown as requirements for normal execution of intracellular bacterial elimination. One study by Wang et al. linked in vitro 1,25-dihydroxyvitamin D (or vitamin D) requirement for normal NOD2 function which was measured through the release of stimulated NF-$\kappa$B products and defensin $\beta$2 [155]. The presence of mutations of NOD2 could not be corrected by increasing media levels of vitamin D. The other paper by Leung et al. reported that, in response to the selective modulation of histone acetylation in the NOD2 promoter region by butyrate, an upregulation of Nod2 was observed. The result is a dramatic enhancement in the production of two chemokines, IL-8 and GRO-$\alpha$, in the presence of PGN. However, in its absence, butyrate only had a slight effect on IL-8 concentration without altering the NF-$\kappa$B associated IL-8 promoter region concentration levels. Their results are in agreement with the observation made by Fusunyan and colleagues, such that NF-$\kappa$B suppression by butyrate is an indication that the upregulation of IL-8 must be independent of NF-$\kappa$B-mediated mechanism [78, 100]. Butyrate addition to the in vitro Caco-2 cell line enhanced PGN-mediated IL-8 and GRO-$\alpha$ production. These products also depended on the induction of NF-$\kappa$B as well as PGN [78]. Taken together these two reports outline a molecular model for the interactions between the NOD2 genetic consortium and 2 important environmental variables which impact on normal function. To date there is no information to our knowledge whether these 2 variables, vitamin D and butyrate, serve redundant or synergistic (additive) functions. Until that time the role of butyrate may be essential for appropriate clearance of intracellular bacterial products and innate immunity.

## 6. Dietary Carbohydrates, Symptoms, Pathogenesis

The impact of dietary interventions for the management of IBD has kept abreast of the scientific research outcome in the last two to three decades, albeit that results are less compelling than theory would suggest. Rationales for specific interventions in particular are more defined. For example, the use of anti-inflammatory omega-3 fatty acids seems rational, although outcomes are not satisfactory [172, 173]. In the case of carbohydrates, Gibson and Shepherd argue that distribution and subsequent rapid fermentation of FODMAP molecules predispose the distal small intestinal and colonic lumen to increased intestinal permeability, an underlying factor to the development of CD in genetically susceptible individuals [8]. They have advocated the pathophysiological involvement of FODMAPs in CD as a direct consequence of widespread consumption in Western societies. Excessive exposure of high fructose corn syrups and caloric sweeteners, commonly present in soft drinks and various manufactured food products [174], also appear to correlate with an increase in functional GI symptoms. As well, lactose sensitivity, independent of known genetic lactase status, has now been confirmed in patients with CD [160].

Consumption of FODMAPs exerts osmotic effects by increasing luminal fluid, inducing intestinal distension, altering intestinal contractile patterns, and accelerating transit time [175]. Development of these symptoms leads to the concept of global restriction of all poorly absorbed, rapidly fermentable short-chain carbohydrates as opposed to selectively limiting a few food items [176–178]. FODMAPs aggravate symptoms possibly further by inducing abnormal motility patterns as a consequence of colonic microfloral modification to accommodate the high volume of such consumption [163] or the incompletely evaluated role of intestinally released gut hormones as described with the prebiotic lactulose [179, 180].

*6.1. Effects of Carbohydrate Withdrawal on Microbial Flora.* Early etiological studies of IBD (especially CD) have consistently suggested that high consumption of refined sugar may be an independent risk factor [181–185]. More recent publications, however, have questioned this effect [186–188]. Nevertheless, a possible explanation for this observation has been provided by the proposed prebiotic concept [2]. There are, however, little data on microbial effects of complex carbohydrate withdrawal. Rats restrictive of food for 20 weeks resulted in nonsignificant changes in reduction of total anaerobic microbes and no significant shifts in population species [189]. When rats were fed sucrose or starch in equicaloric amounts for 9 months, no weight changes occurred,

Table 3: Comparison of putative pathogenic mechanisms in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).

| | | |
|---|---|---|
| IBD | (i) Genetic predisposition (extensive) | [39, 131, 140, 150, 152, 156, 157] |
| | (ii) Intestinal microflora alterations | [55–57, 59–61, 65, 124, 158, 159] |
| | (iii) Altered immunity (extensive) | [42–44, 123, 125, 126] |
| | (iv) Altered carbohydrate sensitivity | [160] |
| | (v) Tissue destruction and complications | [50, 124] |
| IBS | (i) Genetic predisposition (exists but not yet worked out) | [161, 162] |
| | (ii) Microflora alterations especially after gastroenteritis | [6, 33, 163–165] |
| | (iii) Altered immune response (variable and mild) | [166] |
| | (iv) Altered carbohydrate sensitivity | [167–170] |
| | (v) No evidence for tissue destruction | [171] |

but the aerobic population increased and ratio of anaerobes to aerobes decreased [190]. Most importantly the total SCFAs production was significantly higher in starch than sucrose-fed rats, although the ratios remained the same.

*6.2. Effects of Carbohydrate Feeding on Microbial Flora.* On the contrary there are abundant data on the effects of poorly digested carbohydrates on microflora. Maldigested carbohydrates in general alter numbers [191] and type of intestinal cells [192], SCFAs production, colonic pH [191, 193], and microbial numbers as well as diversity [194]. As for prebiotics (those maldigested carbohydrates which fit more to the definitions as proposed by Gibson et al. [195]), short-chain (oligofructose) as well as long-chain fructose (inulin) polymers, all of which promoted the production of SCFAs [196], Bifidobacteria and Lactobacilli species in stool [197, 198], and other mucosal-associated microbial species [199].

In the case of IBD, the introduction of fructooligosaccharides or lactulose in healthy rats has demonstrated a combined effect of increased bacterial translocation, epithelial cell proliferation, colonic epithelial injury, and mucin production despite prebiotic consumption [200]. Among rats fed a FODMAP-like diet in conjunction with *Salmonella* species infection, severe colitis developed while only mild colonic inflammation was observed in controls [200].

Furthermore, a number of other published studies have demonstrated the protective role of both traditional prebiotics as well as other maldigested carbohydrates against experimentally-induced colitis (reviewed in [5]). The animal models employed in those experiments include the IL-10-deficient and trinitrobenzene sulfonic acid (TNBS) mouse model of CD and the DSS mouse model of UC. In these cases lactulose, fructo-oligosaccharide, and trans-galacto-oligosaccharide prebiotics as well as germinated barley foodstuffs (derived from beer production) alter colonic physiology via pH, SCFAs production, microbial species, and outcome of induced colitis.

Probiotics and prebiotics have generally been associated with improvement in clinical IBD [201]. It is postulated that pro- and prebiotics modulate the extent of inflammation during the progressive stage of the condition. In this context probiotics may have an advantage in UC [202], despite benefit of any specific probiotic in CD to date has not been substantiated [203]. Prebiotics in CD have generally shown some effect but again not substantiated (see the following).

*6.3. Irritable Bowel Syndrome and Inflammatory Bowel Disease.* An example where dietary intervention takes into consideration both outlined concepts of carbohydrate effects is IBS. Neurological disturbances [204–207], abnormalities in the brain-gut axis [208, 209], hyperreactivity to stress [210], and impaired gut motility or transit [211, 212] are etiological factors previously proposed to drive symptom profiles of IBS. However, until recently etiological explanations have begun to resemble those of IBD. Genetic factors [161], altered enteric microbiota [164], with a variation of additional bacterial overgrowth in the small intestine [33], and the role of host-microbial communications are gaining importance [6, 7, 162, 165, 213]. High production of acetate and propionate have been observed in correlation with more severe IBS symptoms in patients as reported by Tana et al. [214]. Response to selective probiotics in IBS has also been reported albeit with variable success [10]. While there may be some increased inflammatory cells found on histopathology [166, 215], in cases of postinfection, there is no tissue destruction as seen in IBD. Table 3 outlines some of these similarities.

In general, symptoms in active IBD are attributed to inflammatory processes. However, a fraction of patients defined by clinical criteria to be in remission, nevertheless suffer symptoms which are reminiscent of IBS and satisfy Rome II or III criteria [216]. The use of classical anti-inflammatory medication (e.g., corticosteroids, immunomodulators, etc.) does not seem to alleviate these symptoms and may affect up to a third of patients [216]. Nonetheless, evaluation of fecal calprotectin (a protein marker of true inflammation) in such patients shows elevated levels supporting the notion that these IBS-like symptoms may also be mediated by inflammation. Generally, calprotectin levels are expected to be normal in classical IBS [217].

At present, therapeutic developments targeting those factors remain a complicated task due to the heterogeneity within and among individuals. Unlike IBD, which has a defined immunological pathology, IBS is a highly subjective disorder where hypersensitivity to foodstuffs is mistakenly

perceived by patients as the primary symptomatic factor (the common ill-perceived food constituents are ones originating from dairy products, fructose and wheat products) [167, 168, 208]. Carbohydrate ingestion, in particular, are often avoided. There are thus two approaches to reduce the symptoms as a result of carbohydrate ingestion. Both will be discussed in the following.

## 7. Concepts of Carbohydrates and Therapy: FODMAP Withdrawal Approach

*7.1. Irritable Bowel Syndrome.* After having incorporated FODMAPs as part of their daily diet, subjects (those with preexisting IBS, quiescent IBD condition, or free of intestinal diseases) across several studies had all experienced an increase in effluent load, diarrhea secondary to altered bowel/motility movements, and an overall exacerbation of abdominal symptoms (i.e., flatulence, pain, and bloating) [169, 178]. Contrarily, results derived from other studies involving the restriction of one or more FODMAP food items all showed an improvement of abdominal symptoms in IBS patients [170, 218].

Twelve participants who had previously undergone ileostomy were subjected to either a high or low FODMAP diet for a 4-day period [178]. A 20% increase in the ileal effluent was observed after the participants consumed a high FODMAP diet compared to low, taken into account water content and dry weight also. The effluent consistency was reportedly thicker for the low FODMAP diet as opposed to the high FODMAP diet. Such changes to the nature of ileostomy output are likely influenced by the osmotically active FODMAP components.

Isolated fructose restriction for IBS patients with fructose malabsorption also demonstrated a sustained improvement of functional gut symptoms [218]. In a randomized placebo-controlled crossover trial, fructose, fructans (these are linear or branched polymers of fructose) and a mixture of the two substrates were randomly reintroduced to the original low FODMAP test diet given to a group of fructose malabsorbers with some form of known IBS condition [169]. Despite responding well to the low FODMAP diet for the 10-day duration, 70% of these patients reported symptom recurrence (i.e., diarrhea, abdominal pain, wind, bloating, etc.) upon having their daily meal challenged with fructose and/or fructans in a dose-dependent manner compared to only 14% who received glucose (control). In addition, fructose and fructan combined promoted the greatest symptom severity than either substance alone. This study further supports the dietary principles of FODMAP withdrawal and demonstrates how eliminating the right dietary component is critical to correct IBS symptoms.

It is postulated that the many symptoms (especially diarrhea) felt by IBS patients may be more related to abnormal colonic fermentation rather than osmotic effects, possibly a result of antibiotic- or gastroenteritis-induced dysbiosis [163]. One experiment assessed such correlation by measuring the total body excretion of hydrogen and methane gas in a 24-hour calorimetric test [219]. A comparison between healthy and symptomatic IBS subjects, each consuming two types of diet—a standard fiber-rich and fiber-free diet—found that a significant improvement in abdominal symptoms is in fact associated with the reduction of gaseous products from fiber-free consumption.

Ong and colleagues conducted a randomized, single-blinded, crossover trial to evaluate the impact FODMAP consumption has on the extent and spectrum of intraluminal gas production in 15 healthy volunteers compared to 15 IBS patients by Rome III criteria [220]. Breath hydrogen excretion levels remained fairly high in both groups after a 2-day high FODMAP diet. They observed that those subjected to a high FODMAP diet have a significantly higher incidence of symptoms associated with luminal extension. Interestingly, those without IBS criteria also reported increase in gas production when subjected to a high FODMAP diet, but it did not translate to IBS-related symptoms [220]. Thus, these results indicate that FODMAPs do not cause IBS but that symptoms are triggered by the exaggerated bowel response to gaseous distension [169, 220]. Another study from the UK confirmed the benefit of a low FODMAP diet in IBS patients [221]. Staudacher et al. conducted a diet questionnaire in 82 patients with IBS where they were roughly divided into equal proportions to consume either a standard or a low FODMAP diet. Both groups showed significant improvements in the overall and specific symptoms (e.g., bloating).

*7.2. Inflammatory Bowel Disease.* In the case of IBD, little information is available concerning the specific trials involving carbohydrate restriction. The use of elemental/enteral diets particularly in children to induce CD remission has been explored, but it involves the restriction of most elements from reaching the lower intestine [222, 223]. A randomized controlled trial of carbohydrate restriction was reported by Lorenz-Meyer et al. after 15 years of study [224]. They found some benefit to prevention of relapse in patients with CD, but intention to treat analysis failed to reach significance. More recently, FODMAP withdrawal was reported in a pilot study of 72 patients (52 CD, 20 UC) over a 3-month period [225]. Out of about 70% diet-adherent patients, 50% responded favourably with reductions in abdominal symptoms.

## 8. Concepts of Carbohydrates and Therapy: Emphasis on Prebiotics

*8.1. Irritable Bowel Syndrome.* In contradistinction to FODMAP withdrawal diet, regular consumption of single or mixtures of prebiotics has also been explored for IBS in a few studies. The concept that symptoms of carbohydrate intolerance in healthy persons can be overcome by regular short-term ingestion was observed in populations with lactose intolerance [246]. A formal randomized crossover study of lactose feeding in lactose maldigesters demonstrated both symptomatic and fecal microfloral adaptation [247]. Although symptomatic improvement of lactose intolerance may be due to a placebo effect [248], changes in hydrogen and fecal bacteria are physiological [249–251].

Table 4: (a) Human studies published on the use of prebiotics or nondigestible carbohydrates for inflammatory disorders. IBD: inflammatory bowel disease, UC: ulcerative colitis, CD: Crohn's disease, and P: postoperative ileoanal anastomotic pouch inflammation, represents a spectrum of IBD recurrences. (b) Studies using combination of prebiotics and probiotics (synbiotics) for IBD.

(a)

| Disorder | $N$ = patients | Study type | Active agent | Outcome | Reference |
|---|---|---|---|---|---|
| UC[1] | 29 | RCT | Ispaghula husk | Improved | [226]* |
| UC[1] | 102 | RCT, OL | Plantago Ovata | Nonsuperior | [227] |
| UC[2] | 10 | OL | GBF | Improved | [228] |
| UC[2] | 18 | OL | GBF | Improved | [91] |
| UC[2] | 21 | OL | GBF | Improved | [229] |
| UC[2] | 40 | RCT | GBF | Cytokine decreased | [230] |
| UC[1] | 59 | RCT, OL | GBF | Lower recurrence | [231] |
| UC[2] | 19 | OL | OFS + IN + Bif | Improved clinical endoscopy | [232] |
| UC and CD[1] | 20 (10 controls) | OL | Lactulose | Adaptation in UC, but not in CD | [233] |
| UC and CD[1] | 31 | OL | Lactulose | No effect, but improved quality of life in UC | [234] |
| CD[2] | 10 | OL | FOS, IN | Improved score | [235] |
| CD[2] | 10 | OL | FOS, IN | Improved | [236] |
| CD[2] | 103 | DBRCT | FOS | No clinical benefit, despite impacting on DC function | [237] |
| P[2] | 20 | DBRCT | IN | Improved inflammation | [238]* |
| P[2] | 21 | OL | Lactose | Decreased bacterial sulfomucins | [239] |

(b)

| Disorder | $N$ = patients | Study type | Active agent | Outcome | Reference |
|---|---|---|---|---|---|
| UC[2] | 16 | OL | OFS + IN + Bif | Improved clinical endoscopy | [240] |
| UC[1] | 120 | RCT | Bif/Psy/Bif + Psy | Improved quality of life with Bif + Psy | [241] |
| CD[3] | 30 | OL | Mixed fiber + IN + 4 Lacto | Failed to prevent relapse | [242] |
| CD[2] | 10 | OL | Psy + Bif + Lacto | Clinical improvement | [243] |
| CD[2] | 35 | DBRCT | OFS + IN + Bif | Clinical improvement | [244] |
| P[2] | 10 | OL | OFS, Lacto | Improved and remit | [245] |

RCT: randomized controlled trial; DBRCT: double-blind randomized controlled trial; OL: open labeled; GBF: germinated barley foodstuffs; FOS: fructo-oligosaccharides (<5 degrees of polymerization); OFS: oligofructose (5–10 degrees of polymerization); IN: inulin (<200 degrees of polymerization); Psy: psyllium; Bif: Bifidobacteria species; Lacto: Lactobacillus species.

*Crossover design,

[1]Disease in remission,

[2]Active disease,

[3]Maintenance after surgery.

While it is well recognized that prebiotics induce symptoms in patients, there are now two controlled trials in patients with IBS which demonstrated symptomatic "adaptation" to prolonged feeding. Paineau et al. published a double-blind randomized controlled trial using short-chain fructo-oligosaccharides in 105 patients and reported a global, yet highly specific, symptomatic improvement by the end of the 6-week trial [252]. Similarly, trans-galacto-oligosaccharides employed by Silk et al. in a crossover trial of 44 patients over 12 weeks also reported global and specific improvements [253]. These two studies demonstrate that it is possible to improve symptoms in IBS simply by providing prebiotics on a continual basis. It is not, however, clear whether such improvements were due to "psychological adaption" or bacterial adaptation to carbohydrates.

*8.2. Inflammatory Bowel Disease.* Several studies examining the possible benefits of classical prebiotics (fructose or galactosyl polymers) and poorly digested fibers (e.g., Ispaghula husk, germinated barley foodstuffs) to IBD have been published. The rationale as outlined rests on their ability to modulate the intestinal microflora and their beneficial consequences associated with SCFAs production [91, 206]. These studies comprised of 744 patients with UC, CD, or P (postoperative ileoanal anastomotic pouch inflammation). The variety of indications is described in Tables 4(a) and 4(b), and includes maintenance of remission [226, 227, 231, 234], mild to moderately active disease [91, 228–230, 232, 236–240, 244], prevention of postsurgical CD recurrence [226, 242], and physiological assessment of adaptation capability [233]. The studies include 8 randomized controlled trials of which 3 were double blinded [237, 238, 244] and two were crossover design [226, 238]. The studies extended from 2 weeks to 24 months (mean 4.8 ± 6.1 months, with a median of 1.6 months). A total of 510 patients were treated with active agent and 234 were controls. Of the controls 31 patients received probiotics without prebiotics [241]. Forty-nine treated patients were crossed over to placebo

[226, 238]. While endpoints varied, only two studies failed to show benefit. Six of the randomized studies (4 for UC in remission [226, 227, 241], one active CD [244], and one active P [238]) showed better or nonsuperior remission rates for UC, also improvement in clinical score for CD or P. A small study showed reduction of proinflammatory cytokines in UC [230]. However, the studies failing to show benefit included the largest and most carefully conducted DBRCT (double-blind randomized controlled trial) of patients with active CD [237]. Importantly, it also included the only study albeit observational, evaluating the role of synbiotics in CD postsurgery recurrence [242]. Additional well-conducted trials are needed to lend clinical credence to effective use of prebiotics in IBD.

## 9. Summary and Conclusions

The basic premise of this paper is a conceptual contrast of the rationale of either using a select group of prebiotic molecules to alter microflora and microbial metabolism or to withhold a wide array of carbohydrates which includes those prebiotics. The emphasis of these interventions is on use in IBD, but IBS is used as a clinical model to outline available but to date limited number of trials to show symptomatic efficacy. The two principles pose a scientific conundrum particularly in IBD, while there is evidence that bacterial immune interactions play a significant role in IBS abnormal immune response in IBD lead to tissue destruction.

There is limited evidence that both approaches (withhold FODMAP entirely or use selective parts of FODMAP) in IBS result in symptomatic improvement in a significant percentage of patients within a certain time frame. The use of prebiotics in IBD is not settled in either active or remitting disease. Information on the use of FODMAP or general carbohydrate withdrawal, to our knowledge, has been limited with IBD. The IBS-like symptoms in IBD may be related to intestinal inflammation making its pathogenesis similar but different from that in true IBS. As such the role of beneficial bacteria and SCFAs may be more important in the former.

The real "conundrum," then, is whether the additive or withdrawal approach can induce microbial changes which subsequently lead to amelioration of symptoms (as in IBS or IBS-like symptoms in IBD), but also modulation of the immune response especially inflammation. If both approaches affect the microflora, what organisms are (equally?) modulated by a reduction in specific nutrition as well as kept in check by other organisms like lactic acid-producing bacteria? There is limited research on effects of withdrawal (whether total nutrient or specific nutrients like carbohydrates). There are many publications on effects of addition of prebiotics or complex fibers. The example of NOD2 suggests that certain dietary components may be necessary for normal function, but redundant functions are likely. Nevertheless, until more information is available, a judicious use of the discussed approaches and time of use should be considered for symptom control, with withdrawal (the less tried approach) for IBD.

## References


[1] *The American Heritage Dictionary of the English Language*, Houghton Mifflin, Boston, Mass, USA, 2000.

[2] G. R. Gibson and M. B. Roberfroid, "Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics," *Journal of Nutrition*, vol. 125, no. 6, pp. 1401–1412, 1995.

[3] C. Hedin, K. Whelan, and J. O. Lindsay, "Evidence for the use of probiotics and prebiotics in inflammatory bowel disease: a review of clinical trials," *Proceedings of the Nutrition Society*, vol. 66, no. 3, pp. 307–315, 2007.

[4] C. H. M. Leenen and L. A. Dieleman, "Inulin and oligofructose in chronic inflammatory bowel disease," *Journal of Nutrition*, vol. 137, no. 11, pp. 2572S–2575S, 2007.

[5] A. Szilagyi, "Use of prebiotics for inflammatory bowel disease," *Canadian Journal of Gastroenterology*, vol. 19, no. 8, pp. 505–510, 2005.

[6] E. M. M. Quigley, "Bacterial flora in irritable bowel syndrome: role in pathophysiology, implications for management," *Journal of Digestive Diseases*, vol. 8, no. 1, pp. 2–7, 2007.

[7] U. C. Ghoshal, H. Park, and K. A. Gwee, "Bugs and irritable bowel syndrome: the good, the bad and the ugly," *Journal of Gastroenterology and Hepatology*, vol. 25, no. 2, pp. 244–251, 2010.

[8] P. R. Gibson and S. J. Shepherd, "Personal view: food for thought—western lifestyle and susceptibility to Crohn's disease. The FODMAP hypothesis," *Alimentary Pharmacology and Therapeutics*, vol. 21, no. 12, pp. 1399–1409, 2005.

[9] E. G. Gottschall, *Breaking the Vicious Cycle: Intestinal Health Through Diet*, Kirkton Press, Ontario, Canada, 1994.

[10] G. Aragon, D. B. Graham, M. Borum, and D. B. Doman, "Probiotic therapy for irritable bowel syndrome," *Gastroenterology and Hepatology*, vol. 6, no. 1, pp. 39–44, 2010.

[11] S. L. Greenbloom, A. H. Steinhart, and G. R. Greenberg, "Combination ciprofloxacin and metronidazole for active Crohn's disease," *Canadian Journal of Gastroenterology*, vol. 12, no. 1, pp. 53–56, 1998.

[12] M. Pimentel, W. Morales, K. Chua et al., "Effects of rifaximin treatment and retreatment in nonconstipated IBS subjects," *Digestive Diseases and Sciences*, vol. 56, no. 7, pp. 2067–2072, 2011.

[13] E. M. M. Quigley, "Therapies aimed at the gut microbiota and inflammation: antibiotics, prebiotics, probiotics, synbiotics, anti-inflammatory therapies," *Gastroenterology Clinics of North America*, vol. 40, no. 1, pp. 207–222, 2011.

[14] S. Tedelind, F. Westberg, M. Kjerrulf, and A. Vidal, "Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 13, no. 20, pp. 2826–2832, 2007.

[15] N. Rajendran and D. Kumar, "Role of diet in the management of inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 16, no. 12, pp. 1442–1448, 2010.

[16] C. Palmer, E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown, "Development of the human infant intestinal microbiota," *PLoS Biology*, vol. 5, no. 7, p. e177, 2007.

[17] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Microbiology: diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[18] J. Qin, R. Li, J. Raes et al., "A human gut microbial gene catalogue established by metagenomic sequencing," *Nature*, vol. 464, no. 7285, pp. 59–65, 2010.

[19] L. V. Hooper, T. Midwedt, and J. I. Gordon, "How host-microbial interactions shape the nutrient environment of the mammalian intestine," *Annual Review of Nutrition*, vol. 22, pp. 283–307, 2002.

[20] S. Macfarlane, E. Furrie, A. Kennedy, J. H. Cummings, and G. T. Macfarlane, "Mucosal bacteria in ulcerative colitis," *British Journal of Nutrition*, vol. 93, supplement 1, pp. S67–S72, 2005.

[21] S. Macfarlane and G. T. Macfarlane, "Regulation of short-chain fatty acid production," *Proceedings of the Nutrition Society*, vol. 62, no. 1, pp. 67–72, 2003.

[22] D. J. Morrison, W. G. Mackay, C. A. Edwards, T. Preston, B. Dodson, and L. T. Weaver, "Butyrate production from oligofructose fermentation by the human faecal flora: what is the contribution of extracellular acetate and lactate?" *British Journal of Nutrition*, vol. 96, no. 3, pp. 570–577, 2006.

[23] S. H. Duncan, P. Louis, and H. J. Flint, "Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product," *Applied and Environmental Microbiology*, vol. 70, no. 10, pp. 5810–5817, 2004.

[24] J. H. Cummings and G. T. MacFarlane, "Gastrointestinal effects of prebiotics," *British Journal of Nutrition*, vol. 87, supplement 2, pp. S145–S151, 2002.

[25] C. Duggan, J. Gannon, and W. Allan Walker, "Protective nutrients and functional foods for the gastrointestinal tract," *American Journal of Clinical Nutrition*, vol. 75, no. 5, pp. 789–808, 2002.

[26] D. L. Topping and P. M. Clifton, "Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides," *Physiological Reviews*, vol. 81, no. 3, pp. 1031–1064, 2001.

[27] I. Sekirov, N. M. Tam, M. Jogova et al., "Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection," *Infection and Immunity*, vol. 76, no. 10, pp. 4726–4736, 2008.

[28] I. Sekirov and B. B. Finlay, "The role of the intestinal microbiota in enteric infection," *Journal of Physiology*, vol. 587, no. 17, pp. 4159–4167, 2009.

[29] S. R. Gill, M. Pop, R. T. Deboy et al., "Metagenomic analysis of the human distal gut microbiome," *Science*, vol. 312, no. 5778, pp. 1355–1359, 2006.

[30] E. S. Klaassens, W. M. De Vos, and E. E. Vaughan, "Metaproteomics approach to study the functionality of the microbiota in the human infant gastrointestinal tract," *Applied and Environmental Microbiology*, vol. 73, no. 4, pp. 1388–1392, 2007.

[31] P. J. Turnbaugh, R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon, "The human microbiome project," *Nature*, vol. 449, no. 7164, pp. 804–810, 2007.

[32] C. Manichanh, L. Rigottier-Gois, E. Bonnaud et al., "Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach," *Gut*, vol. 55, no. 2, pp. 205–211, 2006.

[33] E. M. M. Quigley, "Germs, gas and the gut; the evolving role of the enteric flora in IBS," *American Journal of Gastroenterology*, vol. 101, no. 2, pp. 334–335, 2006.

[34] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest," *Nature*, vol. 444, no. 7122, pp. 1027–1031, 2006.

[35] H. Tilg, A. R. Moschen, and A. Kaser, "Obesity and the microbiota," *Gastroenterology*, vol. 136, no. 5, pp. 1476–1483, 2009.

[36] G. W. Sewell, D. J. Marks, and A. W. Segal, "The immunopathogenesis of Crohn's disease: a three-stage model," *Current Opinion in Immunology*, vol. 21, no. 5, pp. 506–513, 2009.

[37] L. D. McVay, S. A. Keilbaugh, T. M. H. Wong et al., "Absence of bacterially induced RELM$\beta$ reduces injury in the dextran sodium sulfate model of colitis," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 2914–2923, 2006.

[38] M. A. McGuckin, R. Eri, L. A. Simms, T. H. J. Florin, and G. Radford-Smith, "Intestinal barrier dysfunction in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 15, no. 1, pp. 100–113, 2009.

[39] C. Abraham and J. H. Cho, "Functional consequences of NOD2 (CARD15) mutations," *Inflammatory Bowel Diseases*, vol. 12, no. 7, pp. 641–650, 2006.

[40] M. Hedl, J. Li, J. H. Cho, and C. Abraham, "Chronic stimulation of Nod2 mediates tolerance to bacterial products," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 49, pp. 19440–19445, 2007.

[41] S. Nell, S. Suerbaum, and C. Josenhans, "The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models," *Nature Reviews Microbiology*, vol. 8, no. 8, pp. 564–577, 2010.

[42] D. J. Marks, M. W. Harbord, R. MacAllister et al., "Defective acute inflammation in Crohn's disease: a clinical investigation," *The Lancet*, vol. 367, no. 9511, pp. 668–678, 2006.

[43] A. M. Smith, F. Z. Rahman, B. Hayee et al., "Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease," *Journal of Experimental Medicine*, vol. 206, no. 9, pp. 1883–1897, 2009.

[44] A. W. Segal and G. Loewi, "Neutrophil dysfunction in Crohn's disease," *The Lancet*, vol. 2, no. 7979, pp. 219–221, 1976.

[45] S. Danese and C. Fiocchi, "Ulcerative colitis," *The New England Journal of Medicine*, vol. 365, no. 18, pp. 1713–1725, 2011.

[46] J. Wehkamp, K. Fellermann, and E. F. Stange, "Human defensins in Crohn's disease: a molecular link to mucosal barrier dysfunction," *Chemical Immunology and Allergy*, vol. 86, pp. 42–54, 2005.

[47] L. A. Simms, J. D. Doecke, M. D. Walsh, N. Huang, E. V. Fowler, and G. L. Radford-Smith, "Reduced $\alpha$-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease," *Gut*, vol. 57, no. 7, pp. 903–910, 2008.

[48] S. Schmechel, A. Konrad, J. Diegelmann et al., "Linking genetic susceptibility to Crohn's disease with Th17 cell function: IL-22 serum levels are increased in Crohn's disease and correlate with disease activity and IL23R genotype status," *Inflammatory Bowel Diseases*, vol. 14, no. 2, pp. 204–212, 2008.

[49] T. Kobayashi, S. Okamoto, T. Hisamatsu et al., "IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease," *Gut*, vol. 57, no. 12, pp. 1682–1689, 2008.

[50] C. Abraham and J. H. Cho, "Inflammatory bowel disease," *New England Journal of Medicine*, vol. 361, no. 21, pp. 2066–2078, 2009.

[51] D. C. Baumgart and S. R. Carding, "Inflammatory bowel disease: cause and immunobiology," *The Lancet*, vol. 369, no. 9573, pp. 1627–1640, 2007.

[52] D. Artis, "Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut,"

*Nature Reviews Immunology*, vol. 8, no. 6, pp. 411–420, 2008.

[53] I. I. Ivanov and D. R. Littman, "Modulation of immune homeostasis by commensal bacteria," *Current Opinion in Microbiology*, vol. 14, no. 1, pp. 106–114, 2011.

[54] J. L. Round and S. K. Mazmanian, "The gut microbiota shapes intestinal immune responses during health and disease," *Nature Reviews Immunology*, vol. 9, no. 5, pp. 313–323, 2009.

[55] A. S. Neish, "Microbes in gastrointestinal health and disease," *Gastroenterology*, vol. 136, no. 1, pp. 65–80, 2009.

[56] R. B. Sartor and M. Muehlbauer, "Microbial host interactions in IBD: implications for pathogenesis and therapy," *Current Gastroenterology Reports*, vol. 9, no. 6, pp. 497–507, 2007.

[57] C. P. Tamboli, C. Neut, P. Desreumaux, and J. F. Colombel, "Dysbiosis in inflammatory bowel disease," *Gut*, vol. 53, no. 1, pp. 1–4, 2004.

[58] P. D. Scanlan, F. Shanahan, C. O'Mahony, and J. R. Marchesi, "Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease," *Journal of Clinical Microbiology*, vol. 44, no. 11, pp. 3980–3988, 2006.

[59] D. N. Frank, A. L. S. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace, "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 34, pp. 13780–13785, 2007.

[60] C. Favier, C. Neut, C. Mizon, A. Cortot, J. F. Colombel, and J. Mizon, "Fecal $\beta$-D-galactosidase production and Bifidobacteria are decreased in Crohn's disease," *Digestive Diseases and Sciences*, vol. 42, no. 4, pp. 817–822, 1997.

[61] P. B. Eckburg and D. A. Relman, "The role of microbes in Crohn's disease," *Clinical Infectious Diseases*, vol. 44, no. 2, pp. 256–262, 2007.

[62] A. Swidsinski, V. Loening-Baucke, M. Vaneechoutte, and Y. Doerffel, "Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora," *Inflammatory Bowel Diseases*, vol. 14, no. 2, pp. 147–161, 2008.

[63] A. Suau, V. Rochet, A. Sghir et al., "Fusobacterium prausnitzii and related species represent a dominant group within the human fecal flora," *Systematic and Applied Microbiology*, vol. 24, no. 1, pp. 139–145, 2001.

[64] A. Swidsinski, V. Loening-Baucke, H. Lochs, and L. P. Hale, "Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice," *World Journal of Gastroenterology*, vol. 11, no. 8, pp. 1131–1140, 2005.

[65] H. M. Martin, B. J. Campbell, C. A. Hart et al., "Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer," *Gastroenterology*, vol. 127, no. 1, pp. 80–93, 2004.

[66] M. H. Giaffer, C. D. Holdsworth, and B. I. Duerden, "Virulence properties of Escherichia coli strains isolated from patients with inflammatory bowel disease," *Gut*, vol. 33, no. 5, pp. 646–650, 1992.

[67] N. Barnich, F. A. Carvalho, A. L. Glasser et al., "CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease," *Journal of Clinical Investigation*, vol. 117, no. 6, pp. 1566–1574, 2007.

[68] B. P. Willing, J. Dicksved, J. Halfvarson et al., "A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes," *Gastroenterology*, vol. 139, no. 6, pp. 1844–1854.e1, 2010.

[69] B. Stecher, S. Chaffron, R. Käppeli et al., "Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria," *PLoS Pathogens*, vol. 6, no. 1, Article ID e1000711, 2010.

[70] A. Macpherson, U. Y. Khoo, I. Forgacs, J. Philpott-Howard, and I. Bjarnason, "Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria," *Gut*, vol. 38, no. 3, pp. 365–375, 1996.

[71] C. O. Elson, "Commensal bacteria as targets in Crohn's disease," *Gastroenterology*, vol. 119, no. 1, pp. 254–257, 2000.

[72] R. Soret, J. Chevalier, P. De Coppet et al., "Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats," *Gastroenterology*, vol. 138, no. 5, pp. 1772–1782.e4, 2010.

[73] H. M. Hamer, D. M. A. E. Jonkers, A. Bast et al., "Butyrate modulates oxidative stress in the colonic mucosa of healthy humans," *Clinical Nutrition*, vol. 28, no. 1, pp. 88–93, 2009.

[74] S. A. L. W. Vanhoutvin, F. J. Troost, H. M. Hamer et al., "Butyrate-induced transcriptional changes in human colonic mucosa," *PLoS One*, vol. 4, no. 8, Article ID e6759, 2009.

[75] K. Daly and S. P. Shirazi-Beechey, "Microarray analysis of butyrate regulated genes in colonic epithelial cells," *DNA and Cell Biology*, vol. 25, no. 1, pp. 49–62, 2006.

[76] J. R. Davie, "Inhibition of histone deacetylase activity by butyrate," *Journal of Nutrition*, vol. 133, no. 7, pp. 2485S–2493S, 2003.

[77] J. P. Segain, J. P. Galmiche, D. Raingeard De La Blétière et al., "Butyrate inhibits inflammatory responses through NF$\kappa$B inhibition: implications for Crohn's disease," *Gut*, vol. 47, no. 3, pp. 397–403, 2000.

[78] C. H. Leung, W. Lam, D. L. Ma, E. A. Gullen, and Y. C. Cheng, "Butyrate mediates nucleotide-binding and oligomerisation domain (NOD) 2-dependent mucosal immune responses against peptidoglycan," *European Journal of Immunology*, vol. 39, no. 12, pp. 3529–3537, 2009.

[79] M. Stempelj, M. Kedinger, L. Augenlicht, and L. Klampfer, "Essential role of the JAK/STAT1 signaling pathway in the expression of inducible nitric-oxide synthase in intestinal epithelial cells and its regulation by butyrate," *Journal of Biological Chemistry*, vol. 282, no. 13, pp. 9797–9804, 2007.

[80] S. Saegusa, M. Totsuka, S. Kaminogawa, and T. Hosoi, "Candida albicans and Saccharomyces cerevisiae induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid," *FEMS Immunology and Medical Microbiology*, vol. 41, no. 3, pp. 227–235, 2004.

[81] M. Schwab, V. Reynders, S. Loitsch, D. Steinhilber, J. Stein, and O. Schröder, "Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NF$\kappa$B signalling," *Molecular Immunology*, vol. 44, no. 15, pp. 3625–3632, 2007.

[82] M. Kinoshita, Y. Suzuki, and Y. Saito, "Butyrate reduces colonic paracellular permeability by enhancing PPAR$\gamma$ activation," *Biochemical and Biophysical Research Communications*, vol. 293, no. 2, pp. 827–831, 2002.

[83] U. Böcker, O. Yezerskyy, P. Feick et al., "Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression," *International Journal of Colorectal Disease*, vol. 18, no. 1, pp. 25–32, 2003.

[84] H. Chirakkal, S. H. Leech, K. E. Brookes, A. L. Prais, J. S. Waby, and B. M. Corfe, "Upregulation of BAK by butyrate in the colon is associated with increased Sp3 binding," *Oncogene*, vol. 25, no. 54, pp. 7192–7200, 2006.

[85] D. Zgouras, A. Wächtershäuser, D. Frings, and J. Stein, "Butyrate impairs intestinal tumor cell-induced angiogenesis by inhibiting HIF- 1$\alpha$ nuclear translocation," *Biochemical and Biophysical Research Communications*, vol. 300, no. 4, pp. 832–838, 2003.

[86] J. Rodríguez-Salvador, C. Armas-Pineda, M. Perezpeña-Diazconti et al., "Effect of sodium butyrate on pro-matrix metalloproteinase-9 and -2 differential secretion in pediatric tumors and cell lines," *Journal of Experimental and Clinical Cancer Research*, vol. 24, no. 3, pp. 463–473, 2005.

[87] J. M. Mariadason, D. H. Barkla, and P. R. Gibson, "Effect of short-chain fatty acids on paracellular permeability in Caco- 2 intestinal epithelium model," *American Journal of Physiology*, vol. 272, no. 4, pp. G705–G712, 1997.

[88] L. Peng, Z. He, W. Chen, I. R. Holzman, and J. Lin, "Effects of butyrate on intestinal barrier function in a caco-2 cell monolayer model of intestinal barrier," *Pediatric Research*, vol. 61, no. 1, pp. 37–41, 2007.

[89] A. J. Wilson and P. R. Gibson, "Short-chain fatty acids promote the migration of colonic epithelial cells in vitro," *Gastroenterology*, vol. 113, no. 2, pp. 487–496, 1997.

[90] S. K. Mazmanian, J. L. Round, and D. L. Kasper, "A microbial symbiosis factor prevents intestinal inflammatory disease," *Nature*, vol. 453, no. 7195, pp. 620–625, 2008.

[91] O. Kanauchi, T. Suga, M. Tochihara et al., "Treatment of ulcerative colitis by feeding with germinated barley foodstuff: first report of a multicenter open control trial," *Journal of Gastroenterology*, vol. 37, supplement 14, pp. 67–72, 2002.

[92] J. M. Harig, K. H. Soergel, R. A. Komorowski, and C. M. Wood, "Treatment of diversion colitis with short-chain-fatty acid irrigation," *New England Journal of Medicine*, vol. 320, no. 1, pp. 23–28, 1989.

[93] H. Lührs, T. Gerke, J. G. Müller et al., "Butyrate inhibits NF-$\kappa$B activation in lamina propria macrophages of patients with ulcerative colitis," *Scandinavian Journal of Gastroenterology*, vol. 37, no. 4, pp. 458–466, 2002.

[94] E. Le Poul, C. Loison, S. Struyf et al., "Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation," *Journal of Biological Chemistry*, vol. 278, no. 28, pp. 25481–25489, 2003.

[95] K. M. Maslowski, A. T. Vieira, A. Ng et al., "Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43," *Nature*, vol. 461, no. 7268, pp. 1282–1286, 2009.

[96] S. Fukuda, H. Toh, K. Hase et al., "Bifidobacteria can protect from enteropathogenic infection through production of acetate," *Nature*, vol. 469, no. 7331, pp. 543–549, 2011.

[97] P. R. Gibson, "The intracellular target of butyrate's actions: HDAC or HDON'T?" *Gut*, vol. 46, no. 4, pp. 447–448, 2000.

[98] M. S. Inan, R. J. Rasoulpour, L. Yin, A. K. Hubbard, D. W. Rosenberg, and C. Giardina, "The luminal short-chain fatty acid butyrate modulates NF-$\kappa$B activity in a human colonic epithelial cell line," *Gastroenterology*, vol. 118, no. 4, pp. 724–734, 2000.

[99] R. F. Place, E. J. Noonan, and C. Giardina, "HDAC inhibition prevents NF-$\kappa$B activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes I$\kappa$B$\alpha$," *Biochemical Pharmacology*, vol. 70, no. 3, pp. 394–406, 2005.

[100] R. D. Fusunyan, J. J. Quinn, M. Fujimoto, R. P. MacDermott, and I. R. Sanderson, "Butyrate switches the pattern of chemokine secretion by intestinal epithelial cells through histone acetylation," *Molecular Medicine*, vol. 5, no. 9, pp. 631–640, 1999.

[101] M. Weng, W. A. Walker, and I. R. Sanderson, "Butyrate regulates the expression of pathogen-triggered IL-8 in intestinal epithelia," *Pediatric Research*, vol. 62, no. 5, pp. 542–546, 2007.

[102] U. Böcker, T. Nebe, F. Herweck et al., "Butyrate modulates intestinal epithelial cell-mediated neutrophil migration," *Clinical and Experimental Immunology*, vol. 131, no. 1, pp. 53–60, 2003.

[103] S. Toden, A. R. Bird, D. L. Topping, and M. A. Conlon, "Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids," *Cancer Biology and Therapy*, vol. 6, no. 2, pp. 253–258, 2007.

[104] P. Rosignolli, R. Fabianni, A. De Bartolomeo et al., "Protective activity of butyrate on hydrogen peroxide-induced DNA damage in isolated human colonocytes and HT29 tumour cells," *Carcinogenesis*, vol. 22, no. 10, pp. 1675–1680, 2001.

[105] J. J. Malago, J. F. J. G. Koninkx, P. C. J. Tooten, E. A. Van Liere, and J. E. Van Dijk, "Anti-inflammatory properties of heat shock protein 70 and butyrate on Salmonella-induced interleukin-8 secretion in enterocyte-like Caco-2 cells," *Clinical and Experimental Immunology*, vol. 141, no. 1, pp. 62–71, 2005.

[106] A. Venkatraman, B. S. Ramakrishna, R. V. Shaji, N. S. N. Kumar, A. Pulimood, and S. Patra, "Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-$\kappa$B," *American Journal of Physiology*, vol. 285, no. 1, pp. G177–G184, 2003.

[107] L. E. M. Willemsen, M. A. Koetsier, S. J. H. Van Deventer, and E. A. F. Van Tol, "Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin $E_1$ and $E_2$ production by intestinal myofibroblasts," *Gut*, vol. 52, no. 10, pp. 1442–1447, 2003.

[108] E. Gaudier, A. Jarry, H. M. Blottière et al., "Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose," *American Journal of Physiology*, vol. 287, no. 6, pp. G1168–G1174, 2004.

[109] C. Moehle, N. Ackermann, T. Langmann et al., "Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease," *Journal of Molecular Medicine*, vol. 84, no. 12, pp. 1055–1066, 2006.

[110] A. Barcelo, J. Claustre, F. Moro, J. A. Chayvialle, J. C. Cuber, and P. Plaisancié, "Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon," *Gut*, vol. 46, no. 2, pp. 218–224, 2000.

[111] J. Schauber, C. Svanholm, S. Termén et al., "Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways," *Gut*, vol. 52, no. 5, pp. 735–741, 2003.

[112] K. Kato, Y. Ishii, S. Mizuno et al., "Usefulness of rectally administering [1-$^{13}$C]-butyrate for breath test in patients with active and quiescent ulcerative colitis," *Scandinavian Journal of Gastroenterology*, vol. 42, no. 2, pp. 207–214, 2007.

[113] S. Nancey, D. Moussata, I. Graber, S. Claudel, J. C. Saurin, and B. Flourié, "Tumor necrosis factor $\alpha$ reduces butyrate oxidation in vitro in human colonic mucosa: a link from inflammatory process to mucosal damage?" *Inflammatory Bowel Diseases*, vol. 11, no. 6, pp. 559–566, 2005.

[114] P. Marteau, "Probiotics, prebiotics, synbiotics: ecological treatment for inflammatory bowel disease?" *Gut*, vol. 55, no. 12, pp. 1692–1693, 2006.

[115] H. M. Hamer, D. Jonkers, K. Venema, S. Vanhoutvin, F. J. Troost, and R. J. Brummer, "Review article: the role of butyrate on colonic function," *Alimentary Pharmacology and Therapeutics*, vol. 27, no. 2, pp. 104–119, 2008.

[116] I. R. Sanderson, "Dietary modulation of GALT," *Journal of Nutrition*, vol. 137, no. 11, pp. 2557S–2562S, 2007.

[117] W. Scheppach and F. Weiler, "The butyrate story: old wine in new bottles?" *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 7, no. 5, pp. 563–567, 2004.

[118] P. Vernia, M. Di Camillo, V. Marinaro, and R. Caprilli, "Effect of predominant methanogenic flora on the outcome of lactose breath test in irritable bowel syndrome patients," *European Journal of Clinical Nutrition*, vol. 57, no. 9, pp. 1116–1119, 2003.

[119] W. Scheppach, H. Sommer, T. Kirchner et al., "Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis," *Gastroenterology*, vol. 103, no. 1, pp. 51–56, 1992.

[120] J. M. W. Wong, R. De Souza, C. W. C. Kendall, A. Emam, and D. J. A. Jenkins, "Colonic health: fermentation and short chain fatty acids," *Journal of Clinical Gastroenterology*, vol. 40, no. 3, pp. 235–243, 2006.

[121] A. H. Steinhart, T. Hiruki, A. Brzezinski, and J. P. Baker, "Treatment of left-sided ulcerative colitis with butyrate enemas: a controlled trial," *Alimentary Pharmacology and Therapeutics*, vol. 10, no. 5, pp. 729–736, 1996.

[122] R. I. Breuer, K. H. Soergel, B. A. Lashner et al., "Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomised, placebo controlled trial," *Gut*, vol. 40, no. 4, pp. 485–491, 1997.

[123] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease," *Nature*, vol. 448, no. 7152, pp. 427–434, 2007.

[124] P. L. Lakatos, S. Fischer, L. Lakatos, I. Gal, and J. Papp, "Current concept on the pathogenesis of inflammatory bowel disease-crosstalk between genetic and microbial factors: pathogenic bacteria and altered bacterial sensing or changes in mucosal integrity take "tool"?" *World Journal of Gastroenterology*, vol. 12, no. 12, pp. 1829–1841, 2006.

[125] F. Z. Rahman, D. J. B. Marks, B. H. Hayee, A. M. Smith, S. L. Bloom, and A. W. Segal, "Phagocyte dysfunction and inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 14, no. 10, pp. 1443–1452, 2008.

[126] J. R. Korzenik and B. K. Dieckgraefe, "Is Crohn's disease an immunodeficiency? A hypothesis suggesting possible early events in the pathogenesis of Crohn's disease," *Digestive Diseases and Sciences*, vol. 45, no. 6, pp. 1121–1129, 2000.

[127] J. R. Turner, "Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application," *American Journal of Pathology*, vol. 169, no. 6, pp. 1901–1909, 2006.

[128] M. Bruewer, A. Luegering, T. Kucharzik et al., "Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms," *Journal of Immunology*, vol. 171, no. 11, pp. 6164–6172, 2003.

[129] S. Dahan, G. Roda, D. Pinn et al., "Epithelial: lamina propria lymphocyte interactions promote epithelial cell differentiation," *Gastroenterology*, vol. 134, no. 1, pp. 192–203, 2008.

[130] J. Berkes, V. K. Viswanathan, S. D. Savkovic, and G. Hecht, "Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation," *Gut*, vol. 52, no. 3, pp. 439–451, 2003.

[131] R. K. Russell and J. Satsangi, "IBD: a family affair," *Best Practice and Research*, vol. 18, no. 3, pp. 525–539, 2004.

[132] N. Mahmud and D. G. Weir, "The urban diet and Crohn's disease: is there a relationship?" *European Journal of Gastroenterology and Hepatology*, vol. 13, no. 2, pp. 93–95, 2001.

[133] A. Schirbel and C. Fiocchi, "Inflammatory bowel disease: established and evolving considerations on its etiopathogenesis and therapy," *Journal of Digestive Diseases*, vol. 11, no. 5, pp. 266–276, 2010.

[134] L. Mayer, "Evolving paradigms in the pathogenesis of IBD," *Journal of Gastroenterology*, vol. 45, no. 1, pp. 9–16, 2010.

[135] T. Gardenbroek, E. Eshuis, C. Ponsioen, D. Ubbink, G. D'Haens, and W. Bemelman, "The effect of appendectomy on the course of ulcerative colitis: a systematic review," *Colorectal Disease*. In press.

[136] A. Hviid, H. Svanström, and M. Frisch, "Antibiotic use and inflammatory bowel diseases in childhood," *Gut*, vol. 60, no. 1, pp. 49–54, 2011.

[137] N. Inohara, Y. Ogura, A. Fontalba et al., "Host recognition of bacterial muramyl dipeptide mediated through NOD2: implications for Crohn's disease," *Journal of Biological Chemistry*, vol. 278, no. 8, pp. 5509–5512, 2003.

[138] K. S. Kobayashi, M. Chamaillard, Y. Ogura et al., "Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract," *Science*, vol. 307, no. 5710, pp. 731–734, 2005.

[139] J. P. Hugot, M. Chamaillard, H. Zouali et al., "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease," *Nature*, vol. 411, no. 6837, pp. 599–603, 2001.

[140] Y. Ogura, D. K. Bonen, N. Inohara et al., "A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease," *Nature*, vol. 411, no. 6837, pp. 603–606, 2001.

[141] C. Q. Zheng, G. Z. Hu, Z. S. Zeng, L. J. Lin, and G. G. Gu, "Progress in searching for susceptibility gene for inflammatory bowel disease by positional cloning," *World Journal of Gastroenterology*, vol. 9, no. 8, pp. 1646–1656, 2003.

[142] D. K. Bonen, Y. Ogura, D. L. Nicolae et al., "Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan," *Gastroenterology*, vol. 124, no. 1, pp. 140–146, 2003.

[143] J. Li, T. Moran, E. Swanson et al., "Regulation of IL-8 and IL-1$\beta$ expression in Crohn's disease associated NOD2/CARD15 mutations," *Human Molecular Genetics*, vol. 13, no. 16, pp. 1715–1725, 2004.

[144] S. Lesage, H. Zouali, J. P. Cézard et al., "CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease," *American Journal of Human Genetics*, vol. 70, no. 4, pp. 845–857, 2002.

[145] J. P. Hugot, I. Zaccaria, J. Cavanaugh et al., "Prevalence of CARD15/NOD2 mutations in Caucasian healthy people," *American Journal of Gastroenterology*, vol. 102, no. 6, pp. 1259–1267, 2007.

[146] N. Kamada, T. Hisamatsu, S. Okamoto et al., "Unique $CD14^+$ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-$\gamma$ axis," *Journal of Clinical Investigation*, vol. 118, no. 6, pp. 2269–2280, 2008.

[147] J. M. Marinis, C. R. Homer, C. McDonald, and D. W. Abbott, "A novel motif in the Crohn's disease susceptibility protein, NOD2, allows TRAF4 to down-regulate innate immune responses," *Journal of Biological Chemistry*, vol. 286, no. 3, pp. 1938–1950, 2011.

[148] K. Cadwell, T. S. Stappenbeck, and H. W. Virgin, "Role of autophagy and autophagy genes in inflammatory bowel

disease," *Current Topics in Microbiology and Immunology*, vol. 335, no. 1, pp. 141–167, 2009.

[149] D. Schmid and C. Münz, "Innate and adaptive immunity through autophagy," *Immunity*, vol. 27, no. 1, pp. 11–21, 2007.

[150] J. D. Rioux, R. J. Xavier, K. D. Taylor et al., "Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis," *Nature Genetics*, vol. 39, no. 5, pp. 596–604, 2007.

[151] M. Parkes, J. C. Barrett, N. J. Prescott et al., "Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility," *Nature Genetics*, vol. 39, no. 7, pp. 830–832, 2007.

[152] J. Hampe, A. Franke, P. Rosenstiel et al., "A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1," *Nature Genetics*, vol. 39, no. 2, pp. 207–211, 2007.

[153] S. A. McCarroll, A. Huett, P. Kuballa et al., "Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease," *Nature Genetics*, vol. 40, no. 9, pp. 1107–1112, 2008.

[154] E. R. Nimmo, C. Stevens, A. M. Phillips et al., "TLE1 modifies the effects of NOD2 in the pathogenesis of Crohn's disease," *Gastroenterology*, vol. 141, no. 3, pp. 972–981.e2, 2011.

[155] T. T. Wang, B. Dabbas, D. Laperriere et al., "Direct and indirect induction by 1,25-dihydroxyvitamin $D_3$ of the NOD2/CARD15-defensin $\beta$2 innate immune pathway defective in crohn disease," *Journal of Biological Chemistry*, vol. 285, no. 4, pp. 2227–2231, 2010.

[156] S. Ishihara, M. M. Aziz, T. Yuki, H. Kazumori, and Y. Kinoshita, "Inflammatory bowel disease: review from the aspect of genetics," *Journal of Gastroenterology*, vol. 44, no. 11, pp. 1097–1108, 2009.

[157] J. C. Barrett, S. Hansoul, D. L. Nicolae et al., "Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease," *Nature Genetics*, vol. 40, no. 8, pp. 955–962, 2008.

[158] A. Swidsinski, A. Ladhoff, A. Pernthaler et al., "Mucosal flora in inflammatory bowel disease," *Gastroenterology*, vol. 122, no. 1, pp. 44–54, 2002.

[159] S. J. Ott, M. Musfeldt, D. F. Wenderoth et al., "Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease," *Gut*, vol. 53, no. 5, pp. 685–693, 2004.

[160] P. Eadala, S. B. Matthews, J. P. Waud, J. T. Green, and A. K. Campbell, "Association of lactose sensitivity with inflammatory bowel disease—demonstrated by analysis of genetic polymorphism, breath gases and symptoms," *Alimentary Pharmacology and Therapeutics*, vol. 34, no. 7, pp. 735–746, 2011.

[161] M. Camilleri, I. Busciglio, P. Carlson et al., "Candidate genes and sensory functions in health and irritable bowel syndrome," *American Journal of Physiology*, vol. 295, no. 2, pp. G219–G225, 2008.

[162] S. Fukudo and M. Kanazawa, "Gene, environment, and brain gut interactions in irritable bowel syndrome," *Journal of gastroenterology and hepatology*, vol. 26, pp. 110–115, 2011.

[163] T. S. King, M. Elia, and J. O. Hunter, "Abnormal colonic fermentation in irritable bowel syndrome," *The Lancet*, vol. 352, no. 9135, pp. 1187–1189, 1998.

[164] K. J. Lee and J. Tack, "Altered intestinal microbiota in irritable bowel syndrome," *Neurogastroenterology and Motility*, vol. 22, no. 5, pp. 493–498, 2010.

[165] L. O'Mahony, J. Mccarthy, P. Kelly et al., "Lactobacillus and Bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles," *Gastroenterology*, vol. 128, no. 3, pp. 541–551, 2005.

[166] A. P. Weston, W. L. Biddle, P. S. Bhatia, and P. B. Miner, "Terminal ileal mucosal mast cells in irritable bowel syndrome," *Digestive Diseases and Sciences*, vol. 38, no. 9, pp. 1590–1595, 1993.

[167] G. R. Locke, A. R. Zinsmeister, N. J. Talley, S. L. Fett, and L. J. Melton, "Risk factors for irritable bowel syndrome: role of analgesics and food sensitivities," *American Journal of Gastroenterology*, vol. 95, no. 1, pp. 157–165, 2000.

[168] M. Simrén, A. Månsson, A. M. Langkilde et al., "Food-related gastrointestinal symptoms in the irritable bowel syndrome," *Digestion*, vol. 63, no. 2, pp. 108–115, 2001.

[169] S. J. Shepherd, F. C. Parker, J. G. Muir, and P. R. Gibson, "Dietary triggers of abdominal symptoms in patients with irritable bowel syndrome: randomized placebo-controlled evidence," *Clinical Gastroenterology and Hepatology*, vol. 6, no. 7, pp. 765–771, 2008.

[170] F. Fernández-Bañares, M. Rosinach, M. Esteve, M. Forné, J. C. Espinós, and J. Maria Viver, "Sugar malabsorption in functional abdominal bloating: a pilot study on the long-term effect of dietary treatment," *Clinical Nutrition*, vol. 25, no. 5, pp. 824–831, 2006.

[171] D. A. Drossman, L. Chang, N. Bellamy et al., "Severity in irritable bowel syndrome: a rome foundation working team report," *American Journal of Gastroenterology*, vol. 106, no. 10, pp. 1749–1759, 2011.

[172] A. Belluzzi, C. Brignola, M. Campieri, A. Pera, S. Boschi, and M. Miglioli, "Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease," *New England Journal of Medicine*, vol. 334, no. 24, pp. 1557–1560, 1996.

[173] T. Tsujikawa, J. Satoh, K. Uda et al., "Clinical importance of n-3 fatty acid-rich diet and nutritional education for the maintenance of remission in Crohn's disease," *Journal of Gastroenterology*, vol. 35, no. 2, pp. 99–104, 2000.

[174] B. M. Popkin and S. J. Nielsen, "The sweetening of the world's diet," *Obesity Research*, vol. 11, no. 11, pp. 1325–1332, 2003.

[175] S. M. Skoog, A. E. Bharucha, M. Camilleri, D. D. Burton, and A. R. Zinsmeister, "Effects of an osmotically active agent on colonic transit," *Neurogastroenterology and Motility*, vol. 18, no. 4, pp. 300–306, 2006.

[176] P. R. Gibson and S. J. Shepherd, "Evidence-based dietary management of functional gastrointestinal symptoms: the FODMAP approach," *Journal of Gastroenterology and Hepatology*, vol. 25, no. 2, pp. 252–258, 2010.

[177] J. Serra, A. Villoria, F. Azpiroz et al., "Impaired intestinal gas propulsion in manometrically proven dysmotility and in irritable bowel syndrome," *Neurogastroenterology and Motility*, vol. 22, no. 4, pp. 401–406, 2010.

[178] J. S. Barrett, R. B. Gearry, J. G. Muir et al., "Dietary poorly absorbed, short-chain carbohydrates increase delivery of water and fermentable substrates to the proximal colon," *Alimentary Pharmacology and Therapeutics*, vol. 31, no. 8, pp. 874–882, 2010.

[179] A. Ropert, C. Cherbut, C. Roze et al., "Colonic fermentation and proximal gastric tone in humans," *Gastroenterology*, vol. 111, no. 2, pp. 289–296, 1996.

[180] S. P. Sheikh, "Neuropeptide Y and peptide YY: major modulators of gastrointestinal blood flow and function," *American Journal of Physiology*, vol. 261, no. 5, pp. G701–G715, 1991.

[181] J. R. Thornton, P. M. Emmett, and K. W. Heaton, "Diet and Crohn's disease: characteristics of the pre-illness diet," *British Medical Journal*, vol. 2, no. 6193, pp. 762–764, 1979.

[182] K. Silkoff, A. Hallak, L. Yegena et al., "Consumption of refined carbohydrate by patients with Crohn's disease in Tel-Aviv-Yafo," *Postgraduate Medical Journal*, vol. 56, no. 662, pp. 842–846, 1980.

[183] G. Jarnerot, I. Jarnmark, and K. Nilsson, "Consumption of refined sugar by patients with Crohn's disease, ulcerative colitis, or irritable bowel syndrome," *Scandinavian Journal of Gastroenterology*, vol. 18, no. 8, pp. 999–1002, 1983.

[184] B. Katschinski, R. F. A. Logan, M. Edmond, and M. J. S. Langman, "Smoking and sugar intake are separate but interactive risk factors in Crohn's disease," *Gut*, vol. 29, no. 9, pp. 1202–1206, 1988.

[185] A. Tragnone, D. Valpiani, F. Miglio et al., "Dietary habits as risk factors for inflammatory bowel disease," *European Journal of Gastroenterology and Hepatology*, vol. 7, no. 1, pp. 47–51, 1995.

[186] A. Sonnenberg, "Geograhic and temporal variations of sugar and margarine consumption in relation to Crohn's disease," *Digestion*, vol. 41, no. 3, pp. 161–171, 1988.

[187] I. Koutroubakis, O. N. Manousos, S. G. M. Meuwissen, and A. S. Pena, "Environmental risk factors in inflammatory bowel disease," *Hepato-Gastroenterology*, vol. 43, no. 8, pp. 381–393, 1996.

[188] T. Yamamoto, M. Nakahigashi, and A. R. Saniabadi, "Review article: diet and inflammatory bowel disease—epidemiology and treatment," *Alimentary Pharmacology and Therapeutics*, vol. 30, no. 2, pp. 99–112, 2009.

[189] A. L. Henderson, W. W. Cao, R. F. Wang, M. H. Lu, and C. E. Cerniglia, "The effect of food restriction on the composition of intestinal microflora in rats," *Experimental Gerontology*, vol. 33, no. 3, pp. 239–247, 1998.

[190] A. Cresci, C. Orpianesi, S. Silvi, V. Mastrandrea, and P. Dolara, "The effect of sucrose or starch-based diet on short-chain fatty acids and faecal microflora in rats," *Journal of Applied Microbiology*, vol. 86, no. 2, pp. 245–250, 1999.

[191] S. Silvi, C. J. Rumney, A. Cresci, and I. R. Rowland, "Resistant starch modifies gut microflora and microbial metabolism in human flora-associated rats inoculated with faeces from Italian and UK donors," *Journal of Applied Microbiology*, vol. 86, no. 3, pp. 521–530, 1999.

[192] R. Sharma and U. Schumacher, "The diet and gut microflora influence the distribution of enteroendocrine cells in the rat intestine," *Experientia*, vol. 52, no. 7, pp. 664–670, 1996.

[193] A. Gostner, M. Blaut, V. Schäffer et al., "Effect of isomalt consumption on faecal microflora and colonic metabolism in healthy volunteers," *British Journal of Nutrition*, vol. 95, no. 1, pp. 40–50, 2006.

[194] A. Högberg, J. E. Lindberg, T. Leser, and P. Wallgren, "Influence of cereal non-starch polysaccharides on ileo-caecal and rectal microbial populations in growing pigs," *Acta Veterinaria Scandinavica*, vol. 45, no. 1-2, pp. 87–98, 2004.

[195] G. R. Gibson, H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid, "Dietary modulation of the human colonic microbiota: updating the concept of prebiotics," *Nutrition Research Reviews*, vol. 17, no. 2, pp. 259–275, 2004.

[196] B. Kleessen, L. Hartmann, and M. Blaut, "Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora," *British Journal of Nutrition*, vol. 86, no. 2, pp. 291–300, 2001.

[197] Y. Bouhnik, A. Attar, F. A. Joly, M. Riottot, F. Dyard, and B. Flourié, "Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans," *European Journal of Clinical Nutrition*, vol. 58, no. 3, pp. 462–466, 2004.

[198] Y. Bouhnik, L. Raskine, G. Simoneau, D. Paineau, and F. Bornet, "The capacity of short-chain fructo-oligosaccharides to stimulate faecal bifidobacteria: a dose-response relationship study in healthy humans," *Nutrition Journal*, vol. 5, article 8, 2006.

[199] S. J. Langlands, M. J. Hopkins, N. Coleman, and J. H. Cummings, "Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel," *Gut*, vol. 53, no. 11, pp. 1610–1616, 2004.

[200] I. M. J. Bovee-Oudenhoven, S. J. M. Ten Bruggencate, M. L. G. Lettink-Wissink, and R. Van Der Meer, "Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats," *Gut*, vol. 52, no. 11, pp. 1572–1578, 2003.

[201] S. O'Flaherty, D. M. Saulnier, B. Pot, and J. Versalovic, "How can probiotics and prebiotics impact mucosal immunity?" *Gut Microbes*, vol. 1, no. 5, p. 293, 2010.

[202] D. Heilpern and A. Szilagyi, "Manipulation of intestinal microbial flora for therapeutic benefit in inflammatory bowel diseases: review of clinical trials of probiotics, prebiotics and synbiotics," *Reviews on Recent Clinical Trials*, vol. 3, no. 3, pp. 167–184, 2008.

[203] V. E. Rolfe, P. J. Fortun, C. J. Hawkey, and F. Bath-Hextall, "Probiotics for maintenance of remission in Crohn's disease," *Cochrane Database of Systematic Reviews*, no. 4, p. CD004826, 2006.

[204] C. L. Kwan, N. E. Diamant, G. Pope, K. Mikula, D. J. Mikulis, and K. D. Davis, "Abnormal forebrain activity in functional bowel disorder patients with chronic pain," *Neurology*, vol. 65, no. 8, pp. 1268–1277, 2005.

[205] F. Azpiroz, M. Bouin, M. Camilleri et al., "Mechanisms of hypersensitivity in IBS and functional disorders," *Neurogastroenterology and Motility*, vol. 19, supplement 1, pp. 62–88, 2007.

[206] K. Tillisch and E. A. Mayer, "Pain perception in irritable bowel syndrome," *CNS Spectrums*, vol. 10, no. 11, pp. 877–882, 2005.

[207] U. Blankstein, J. Chen, N. E. Diamant, and K. D. Davis, "Altered brain structure in irritable bowel syndrome: potential contributions of pre-existing and disease-driven factors," *Gastroenterology*, vol. 138, no. 5, pp. 1783–1789, 2010.

[208] M. P. Jones, J. B. Dilley, D. Drossman, and M. D. Crowell, "Brain-gut connections in functional GI disorders: anatomic and physiologic relationships," *Neurogastroenterology and Motility*, vol. 18, no. 2, pp. 91–103, 2006.

[209] H. Törnblom, G. Lindberg, B. Nyberg, and B. Veress, "Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome," *Gastroenterology*, vol. 123, no. 6, pp. 1972–1979, 2002.

[210] W. E. Whitehead, M. D. Crowell, J. C. Robinson, B. R. Heller, and M. M. Schuster, "Effects of stressful life events on bowel symptoms: subjects with irritable bowel syndrome compared with subjects without bowel dysfunction," *Gut*, vol. 33, no. 6, pp. 825–830, 1992.

[211] J. E. Kellow, G. M. Eckersley, and M. P. Jones, "Enhanced perception of physiological intestinal motility in the irritable bowel syndrome," *Gastroenterology*, vol. 101, no. 6, pp. 1621–1627, 1991.

[212] J. Serra, F. Azpiroz, and J. R. Malagelada, "Impaired transit and tolerance of intestinal gas in the irritable bowel syndrome," *Gut*, vol. 48, no. 1, pp. 14–19, 2001.

[213] M. Ortiz Lucas, P. Saz Peiró, and J. J. Sebastián Domingo, "Irritable bowel syndrome immune hypothesis. Part two: the role of cytokines," *Revista Espanola de Enfermedades Digestivas*, vol. 102, no. 12, pp. 711–717, 2010.

[214] C. Tana, Y. Umesaki, A. Imaoka, T. Handa, M. Kanazawa, and S. Fukudo, "Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome," *Neurogastroenterology and Motility*, vol. 22, no. 5, pp. 512–519, 2010.

[215] V. S. Chadwick, W. Chen, D. Shu et al., "Activation of the mucosal immune system in irritable bowel syndrome," *Gastroenterology*, vol. 122, no. 7, pp. 1778–1783, 2002.

[216] J. Keohane, C. O'Mahony, L. O'Mahony, S. O'Mahony, E. M. Quigley, and F. Shanahan, "Irritable bowel syndrome-type symptoms in patients with inflammatory bowel disease: a real association or reflection of occult inflammation?" *The American Journal of Gastroenterology*, vol. 105, no. 8, pp. 1788–1795, 2010.

[217] F. Costa, M. G. Mumolo, M. Bellini et al., "Role of faecal calprotectin as non-invasive marker of intestinal inflammation," *Digestive and Liver Disease*, vol. 35, no. 9, pp. 642–647, 2003.

[218] S. J. Shepherd and P. R. Gibson, "Fructose malabsorption and symptoms of irritable bowel syndrome: guidelines for effective dietary management," *Journal of the American Dietetic Association*, vol. 106, no. 10, pp. 1631–1639, 2006.

[219] K. L. E. Dear, M. Elia, and J. O. Hunter, "Do interventions which reduce colonic bacterial fermentation improve symptoms of irritable bowel syndrome?" *Digestive Diseases and Sciences*, vol. 50, no. 4, pp. 758–766, 2005.

[220] D. K. Ong, S. B. Mitchell, J. S. Barrett et al., "Manipulation of dietary short chain carbohydrates alters the pattern of gas production and genesis of symptoms in irritable bowel syndrome," *Journal of Gastroenterology and Hepatology*, vol. 25, no. 8, pp. 1366–1373, 2010.

[221] H. M. Staudacher, K. Whelan, P. M. Irving, and M. C. E. Lomer, "Comparison of symptom response following advice for a diet low in fermentable carbohydrates (FODMAPs) versus standard dietary advice in patients with irritable bowel syndrome," *Journal of Human Nutrition and Dietetics*, vol. 24, no. 5, pp. 487–495, 2011.

[222] F.M. Ruemmele, "Early programming effects of nutrition—life-long consequences?" *Annals of Nutrition and Metabolism*, vol. 58, supplement 2, pp. 5–6, 2011.

[223] F. M. Ruemmele, C. C. Roy, E. Levy, and E. G. Seidman, "Nutrition as primary therapy in pediatric Crohn's disease: fact or fantasy?" *Journal of Pediatrics*, vol. 136, no. 3, pp. 285–291, 2000.

[224] H. Lorenz-Meyer, P. Bauer, C. Nicolay et al., "Omega-3 fatty acids and low carbohydrate diet for maintenance of remission in Crohn's disease. A randomized controlled multicenter trial," *Scandinavian Journal of Gastroenterology*, vol. 31, no. 8, pp. 778–785, 1996.

[225] R. B. Gearry, P. M. Irving, J. S. Barrett, D. M. Nathan, S. J. Shepherd, and P. R. Gibson, "Reduction of dietary poorly absorbed short-chain carbohydrates (FODMAPs) improves abdominal symptoms in patients with inflammatory bowel disease-a pilot study," *Journal of Crohn's and Colitis*, vol. 3, no. 1, pp. 8–14, 2009.

[226] C. Hallert, M. Kaldma, and B. G. Petersson, "Ispaghula husk may relieve gastrointestinal symptoms in ulcerative colitis in remission," *Scandinavian Journal of Gastroenterology*, vol. 26, no. 7, pp. 747–750, 1991.

[227] F. Fernández-Bañares, J. Hinojosa, J. L. Sânchcz-Lombraña et al., "Randomized clinical trial of Plantago ovata seeds (Dietary fiber) as compared with mesalamine in maintaining remission in ulcerative colitis," *American Journal of Gastroenterology*, vol. 94, no. 2, pp. 427–433, 1999.

[228] K. Mitsuyama, T. Saiki, O. Kanauchi et al., "Treatment of ulcerative colitis with germinated barley foodstuff feeding: a pilot study," *Alimentary Pharmacology and Therapeutics*, vol. 12, no. 12, pp. 1225–1230, 1998.

[229] O. Kanauchi, K. Mitsuyama, T. Homma et al., "Treatment of ulcerative colitis patients by long-term administration of germinated barley foodstuff: multi-center open trial," *International Journal of Molecular Medicine*, vol. 12, no. 5, pp. 701–704, 2003.

[230] Z. Faghfoori, L. Navai, R. Shakerhosseini, M. H. Somi, Z. Nikniaz, and M. F. Norouzi, "Effects of an oral supplementation of germinated barley foodstuff on serum tumour necrosis factor-$\alpha$, interleukin-6 and -8 in patients with ulcerative colitis," *Annals of Clinical Biochemistry*, vol. 48, no. 3, pp. 233–237, 2011.

[231] H. Hanai, O. Kanauchi, K. Mitsuyama et al., "Germinated barley foodstuff prolongs remission in patients with ulcerative colitis," *International Journal of Molecular Medicine*, vol. 13, no. 5, pp. 643–647, 2004.

[232] F. Casellas, N. Borruel, A. Torrejón et al., "Oral oligofructose-enriched inulin supplementation in acute ulcerative colitis is well tolerated and associated with lowered faecal calprotectin," *Alimentary Pharmacology and Therapeutics*, vol. 25, no. 9, pp. 1061–1067, 2007.

[233] A. Szilagyi, J. Rivard, and I. Shrier, "Diminished efficacy of colonic adaptation to lactulose occurs in patients with inflammatory bowel disease in remission," *Digestive Diseases and Sciences*, vol. 47, no. 12, pp. 2811–2822, 2002.

[234] A. Hafer, S. Krämer, S. Duncker, M. Krüger, M. P. Manns, and S. C. Bischoff, "Effect of oral lactulose on clinical and immunohistochemical parameters in patients with inflammatory bowel disease: a pilot study," *BMC Gastroenterology*, vol. 7, article 36, 2007.

[235] T. A. Hussey, R. M. Issenman, and S. Persad, "Nutrition therapy in pediatric therapy improves nutrition status and decreases inflammation," *Journal of Pediatric Gastroenterology*, vol. 37, p. 34, 2003.

[236] J. O. Lindsay, K. Whelan, A. J. Stagg et al., "Clinical, microbiological, and immunological effects of fructo-oligosaccharide in patients with Crohn's disease," *Gut*, vol. 55, no. 3, pp. 348–355, 2006.

[237] J. L. Benjamin, C. R.H. Hedin, A. Koutsoumpas et al., "Randomised, double-blind, placebo-controlled trial of fructo-oligosaccharides in active Crohn's disease," *Gut*, vol. 60, no. 7, pp. 923–929, 2011.

[238] C. F. M. Welters, E. Heineman, F. B. J. M. Thunnissen, A. E. J. M. Van den Bogaard, P. B. Soeters, and C. G. M. I. Baeten, "Effect of dietary inulin supplementation on inflammation of pouch mucosa in patients with an ileal pouch-anal anastomosis," *Diseases of the Colon and Rectum*, vol. 45, no. 5, pp. 621–627, 2002.

[239] J. Kuisma, S. Mentula, H. Jarvinen, A. Kahri, M. Saxelin, and M. Farkkila, "Effect of Lactobacillus rhamnosus GG on ileal pouch inflammation and microbial flora," *Alimentary Pharmacology and Therapeutics*, vol. 17, no. 4, pp. 509–515, 2003.

[240] E. Furrie, S. Macfarlane, A. Kennedy et al., "Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial," *Gut*, vol. 54, no. 2, pp. 242–249, 2005.

[241] S. Fujimori, K. Gudis, K. Mitsui et al., "A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis," *Nutrition*, vol. 25, no. 5, pp. 520–525, 2009.

[242] I. Chermesh, A. Tamir, R. Reshef et al., "Failure of synbiotic 2000 to prevent postoperative recurrence of Crohn's disease," *Digestive Diseases and Sciences*, vol. 52, no. 2, pp. 385–389, 2007.

[243] S. Fujimori, A. Tatsuguchi, K. Gudis et al., "High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease," *Journal of Gastroenterology and Hepatology*, vol. 22, no. 8, pp. 1199–1204, 2007.

[244] H. Steed, G. T. MacFarlane, K. L. Blackett et al., "Clinical trial: the microbiological and immunological effects of synbiotic consumption—a randomized double-blind placebo-controlled study in active Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 32, no. 7, pp. 872–883, 2010.

[245] G. Friedman and J. George, "Treatment of refractory pouchitis with prebiotic and probiotic therapy," *Gastroenterology*, vol. 118, p. G4167, 2000.

[246] A. Szilagyi, "Review article: lactose—a potential prebiotic," *Alimentary Pharmacology and Therapeutics*, vol. 16, no. 9, pp. 1591–1602, 2002.

[247] S. R. Hertzler and D. A. Savaiano, "Colonic adaptation to daily lactose feeding in lactose maldigesters reduces lactose intolerance," *American Journal of Clinical Nutrition*, vol. 64, no. 2, pp. 232–236, 1996.

[248] F. Briet, P. Pochart, P. Marteau, B. Flourie, E. Arrigoni, and J. C. Rambaud, "Improved clinical tolerance to chronic lactose ingestion in subjects with lactose intolerance: a placebo effect?" *Gut*, vol. 41, no. 5, pp. 632–715, 1997.

[249] T. Jiang and D. A. Savaiano, "In vitro lactose fermentation by human colonic bacteria is modified by Lactobacillus acidophilus supplementation," *Journal of Nutrition*, vol. 127, no. 8, pp. 1489–1495, 1997.

[250] T. Jiang and D. A. Savaiano, "Modification of colonic fermentation by bifidobacteria and pH In Vitro: impact on lactose metabolism, short-chain fatty acid, and lactate production," *Digestive Diseases and Sciences*, vol. 42, no. 11, pp. 2370–2377, 1997.

[251] H. A. Mäkivuokko, M. T. Saarinen, A. C. Ouwehand, and N. E. Rautonen, "Effects of lactose on colon microbial community structure and function in a four-stage semi-continuous culture system," *Bioscience, Biotechnology and Biochemistry*, vol. 70, no. 9, pp. 2056–2063, 2006.

[252] D. Paineau, F. Payen, S. Panserieu et al., "The effects of regular consumption of short-chain fructo-oligosaccharides on digestive comfort of subjects with minor functional bowel disorders," *British Journal of Nutrition*, vol. 99, no. 2, pp. 311–318, 2008.

[253] D. B. A. Silk, A. Davis, J. Vulevic, G. Tzortzis, and G. R. Gibson, "Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome," *Alimentary Pharmacology and Therapeutics*, vol. 29, no. 5, pp. 508–518, 2009.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 658786, 16 pages
doi:10.1155/2012/658786

*Review Article*

# Development of an Inflammation-Associated Colorectal Cancer Model and Its Application for Research on Carcinogenesis and Chemoprevention

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Received 9 September 2011; Accepted 25 October 2011

Academic Editor: J. Braun



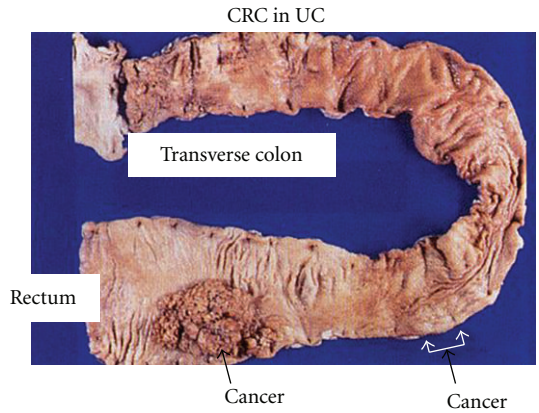
Chronic inflammation is a well-recognized risk factor for development of human cancer in several tissues, including large bowel. Inflammatory bowel disease, including ulcerative colitis and Crohn's disease, is a longstanding inflammatory disease of intestine with increased risk for colorectal cancer development. Several molecular events involved in chronic inflammatory process may contribute to multistep carcinogenesis of human colorectal cancer in the inflamed colon. They include overproduction of reactive oxygen and nitrogen species, overproduction and upregulation of productions and enzymes of arachidonic acid biosynthesis pathway and cytokines, and intestinal immune system dysfunction. In this paper, I will describe several methods to induce colorectal neoplasm in the inflamed colon. First, I will introduce a protocol of a novel inflammation-associated colon carcinogenesis in mice. In addition, powerful tumor-promotion/progression activity of dextran sodium sulfate in the large bowel of $Apc^{Min/+}$ mice will be described. Finally, chemoprevention of inflammation-associated colon carcinogenesis will be mentioned.

## 1. Introduction

Relationship between inflammation and cancer has been suggested for a long time [1]. Since Marshall and Warren [2], who discovered *Helicobacter pylori* and reported its infection closely associated with gastric cancer development, won the Nobel Prize in Physiology or Medicine in 2005, there have been an increasing number of reports on PubMed as to the relationship between inflammation and carcinogenesis in a variety of tissues (Table 1) and it has been featured in major journals.

In terms of the large bowel, it has been found that the risk of colorectal cancer increases in relation to the degrees of inflammation and the disease duration (duration/risk = 10 years/1.6%, 20 years/8.3%, and 30 years/18.4%) in inflammatory bowl diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease (CD) (Figure 1) [3]. I have been interested in inflammation-associated colorectal carcinogenesis for a long time, since even younger patients with UC have high risk of colorectal cancer [4].

Patients with UC as well as those with colorectal cancer have been increasing in Asian countries including Japan, similarly to Western countries (Figure 2) [5]. Therefore, it is necessary to investigate the mechanisms of colorectal cancer development with the background of inflammation for establishing the countermeasure strategy such as chemoprevention [6–8]. To this end, a novel animal model is required but there have been few useful animal models. In this paper, I would like to introduce details of my short-term mouse and rat colorectal cancer models with the background of colitis mimicking human UC and our exploration of chemopreventive agents using these models [6–8].




Rate of CRC development in UC patients:

| Dis. period (year) | Rate (%) |
|---|---|
| 10 | 1.6 |
| 20 | 8.3 |
| 30 | 18.4 |

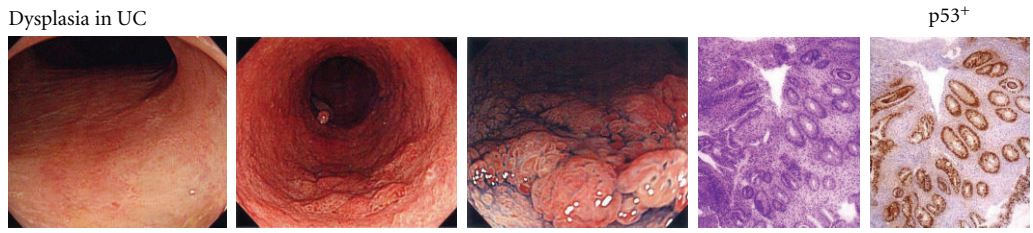


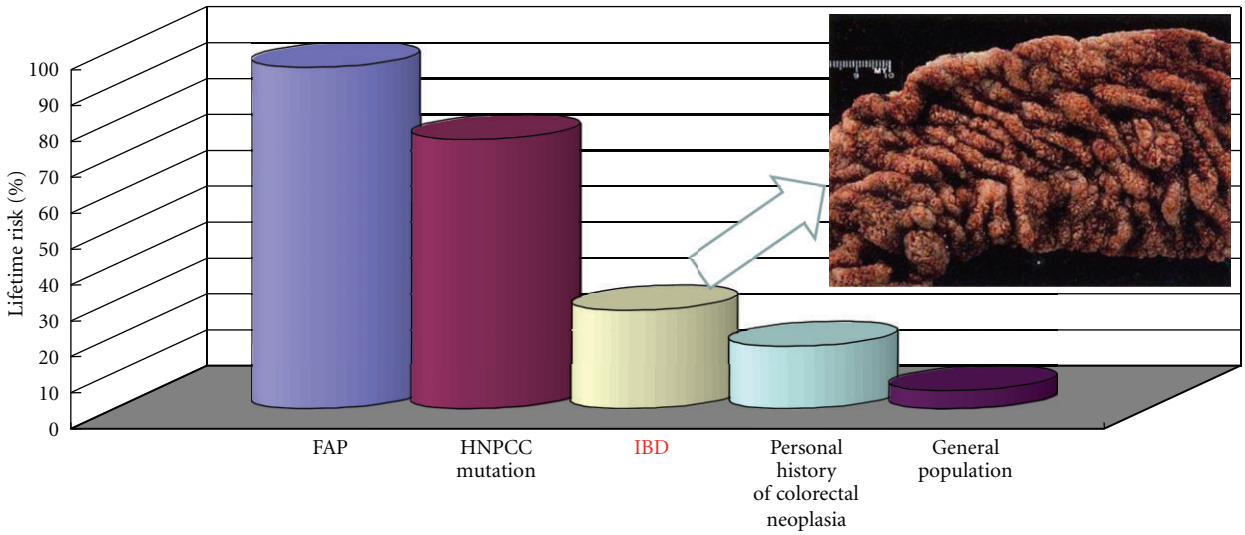

Figure 1: UC patients are high-risk groups of colorectal cancer (CRC) development.

Table 1: Inflammation and cancer in various tissues.

| Chronic inflammation | Site and associated cancer |
|---|---|
| Chewing tobacco, Oral irritation | Oral squamous cell carcinoma |
| Smoking, Chronic bronchitis, Chronic obstructive pulmonary disease | Lung cancer |
| Asbestosis | Mesothelioma |
| Reflux esophagitis, Barrett's esophagus | Esophageal adenocarcinoma |
| *H. pylori*-induced gastritis | Gastric cancer, Mucosa-associated lymphoid tissue lymphoma |
| Chronic pancreatitis | Pancreatic adenocarcinoma |
| Viral (Hepatitis B and C virus) hepatitis | Hepatocellular carcinoma |
| Opisthorchis sinensis infection (liver fluke) | Cholangio carcinoma |
| Inflammatory bowel disease (IBD) | Colorectal adenocarcinoma |
| Pelvic inflammatory disease | Ovarian cancer |
| Human papilloma virus (HPV) infection | Anogenital carcinoma |
| Schistosomiasis | Bladder cancer |
| Chronic scar tissue | Scar cancer arising in pre-existing scars in the lung, skin, and other tissues |
| Human herpes simplex virus type 8 | Kaposi sarcoma |
| Chronic oesteomyelitis | Osteosarcoma |

## 2. Process of Human Colorectal Carcinogenesis

There are at least four types of human colorectal carcinogenesis (adenoma-carcinoma sequence type, hereditary nonpolyposis colorectal cancer (HNPCC) type, *de novo* type, and colitic cancer type) (Figure 3) [9]. Of them, the colitic (colitis-associated) cancer type arises from the background of colitis and DNA injury is induced by production of free radicals by the inducible nitric oxide synthase (iNOS) system in the colonic mucosa with persistent inflammation, followed by *p53* mutation and development of dysplasia, a precancerous lesion. Furthermore, dysplasia is advanced by cyclooxygenase- (COX-) 2, iNOS, and several cytokines produced in the infiltrated inflammatory cells and accumulation of genetic abnormality, such as a loss of the *DCC* gene, leads to invasive colorectal cancer. Unlike common colorectal cancer (adenoma-carcinoma sequence type), it has been thought that the *APC* and K-*ras* genes and microsatellite instability (MSI) are hardly involved in this type, but there remains to be further discussed [9].




UC patients have been increasing in Japan and Korea

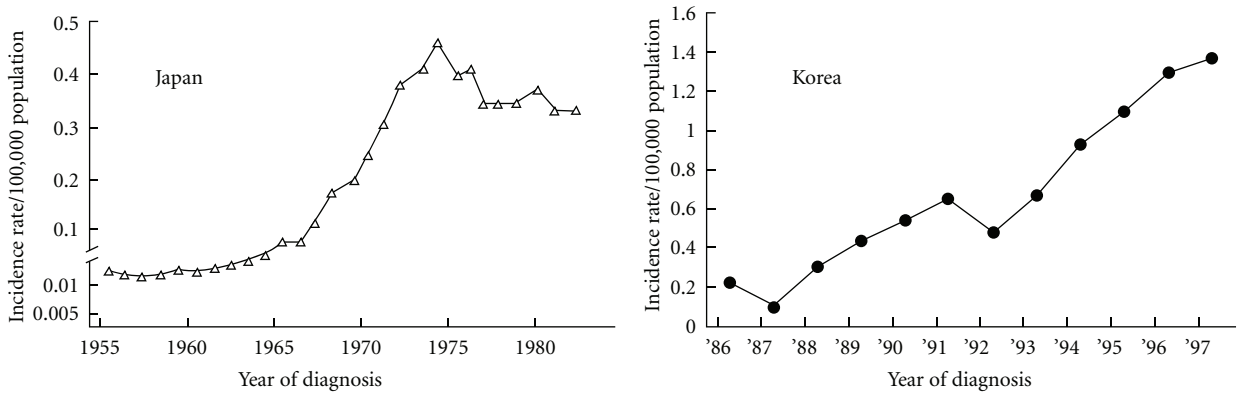



Figure 2: Risk of colorectal cancer.

## 3. Development of an Inflammation-Associated Colorectal Cancer Model

Rats have mostly been employed for an animal colorectal carcinogenesis model, and azoxymethane (AOM), methylazoxymethanol (MAM) acetate, and 1,2-dimethylhydrazine (DMH) have been widely used as colorectal carcinogenic substances (Table 2) [10]. About 30 weeks are required for development of colorectal cancer in about half of rats that are initiated with the colonic carcinogens. On the other hand, in experiments and studies using mice, multiple administrations of similar colorectal carcinogens are required and it takes a long term of 40 weeks or longer to develop colorectal cancer [11]. Therefore, I tried to develop a novel mouse model that would develop colorectal cancer in a short term in the inflamed colon [12]. To settle the issue of the influence of peroxisome proliferator-activated receptor (PPAR) agonists on colorectal carcinogenesis, which has been a topic on the journal *Nat Med* since 1998 [13–15], we confirmed that colitis inducing dextran sodium sulfate (DSS), employed in an experiment using rats with aberrant crypt foci (ACF) as a biological marker (Figure 4) [9, 16–18], had tumor promoter activity to accelerate development of ACF and hypothesized that a combination of DSS and AOM would induce colorectal cancer in a short-term period in mice as well [19].

Since DSS is a nongenotoxic carcinogen [20], male ICR mice were divided into three groups that received different administration patterns: DSS → AOM, AOM during DSS administration, and AOM → DSS (Figure 5). In the groups of DSS → AOM and AOM → DSS, there was a one-week interval between the treatments [12]. DSS was given at the concentration of 2% in drinking water (distilled water) for one week and AOM was administered intraperitoneally once at a low dose of 10 mg/kg body weight, which could not induce colorectal tumors, namely, the low-dose initiation. Interestingly, many colorectal tumors (tubular adenomas

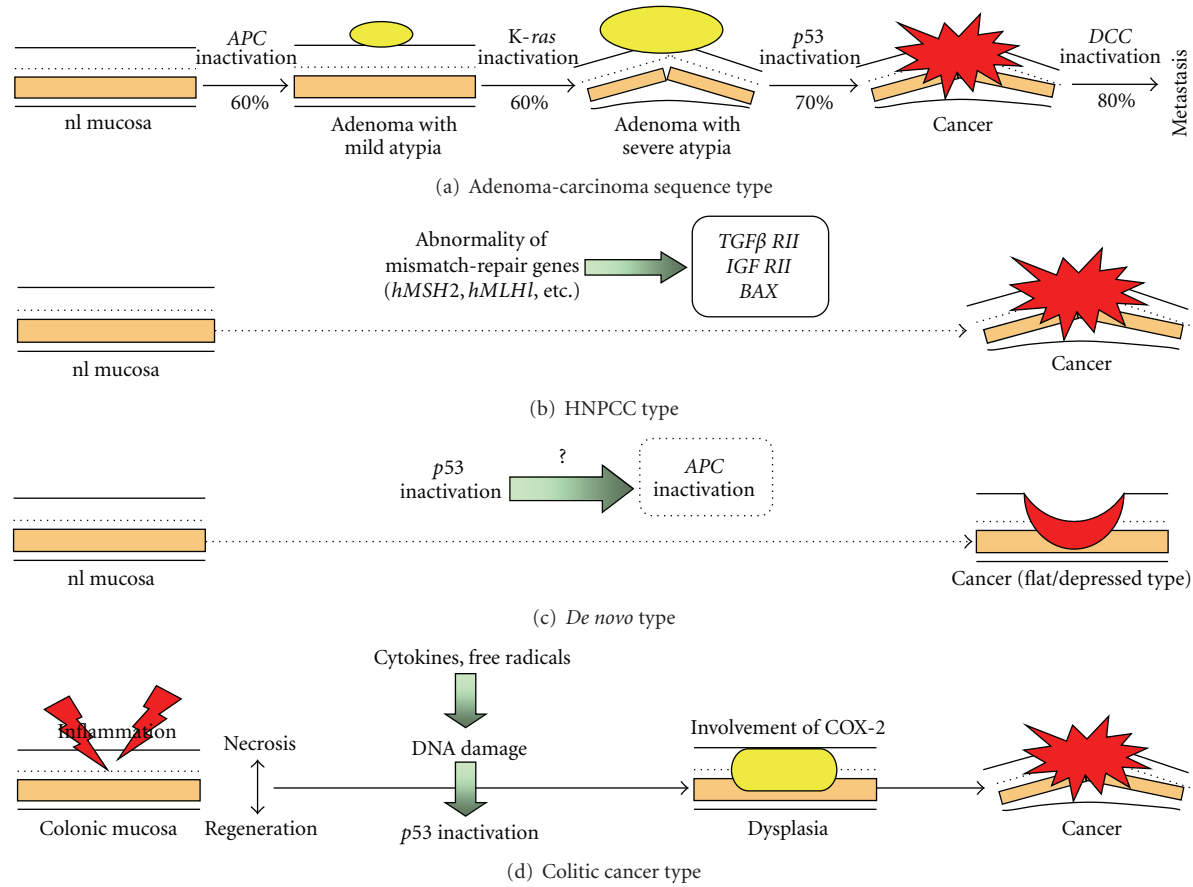



Figure 3: Carcinogenic steps of four types of human colorectal cancer.

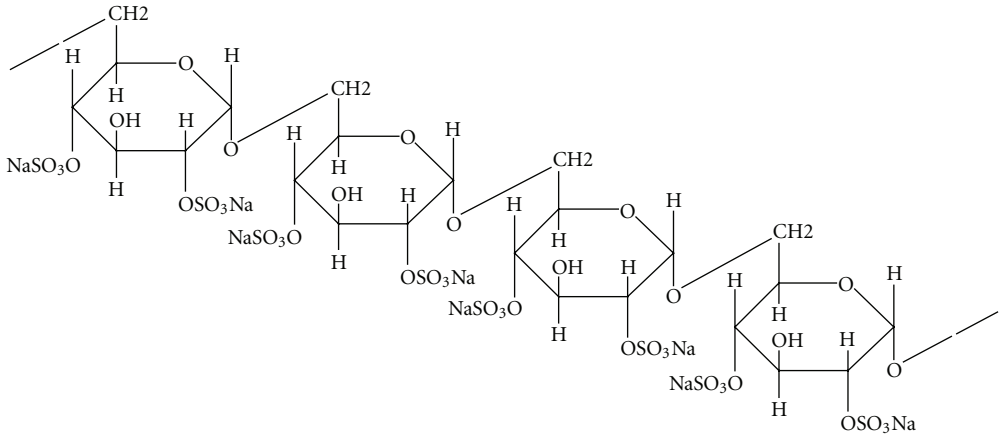



Figure 4: Chemical structure of dextran sulfate sodium (DSS), a sulfated polysaccharide, and its biological activities. DSS (1–5% in drinking water or diet) induces colitis in rodents. Treatment with DSS (1% in diet) after DMH exposure produces colonic adenocarcinoma [44]. The tumorigenicityof DSS is non-genotoxic effects [20]. Cycle treatment with 3% DSS (MW 54,000, 7 days) and distilled water (14 days) produces colonic tumors [45]. DSS increases the number of ACF induced by AOM [19].

and tubular adenocarcinomas) developed in the distal colon, where DSS could induce severe colitis, of mice in the group of AOM → DSS. On the other hand, mice of other groups (the DSS → AOM and the AOM during DSS administration groups) did not develop colorectal tumors. The findings confirm potent tumor-promotion activity of DSS (Figure 6). At the same time, the results reconfirmed importance of inflammation in colorectal carcinogenesis [12]. In addition, accumulation of $\beta$-catenin in the nuclei of colorectal adenocarcinoma cells was observed.

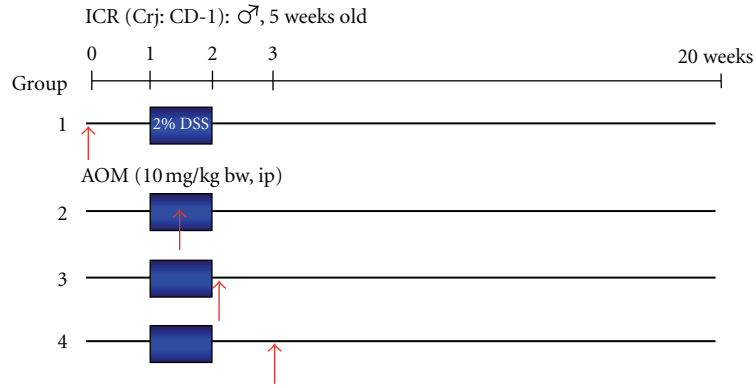



Figure 5: Experimental protocol to develop an inflammation-associated mouse colon carcinogenesis model, to develop a new inflammation-related mouse colon carcinogenesis model [12].

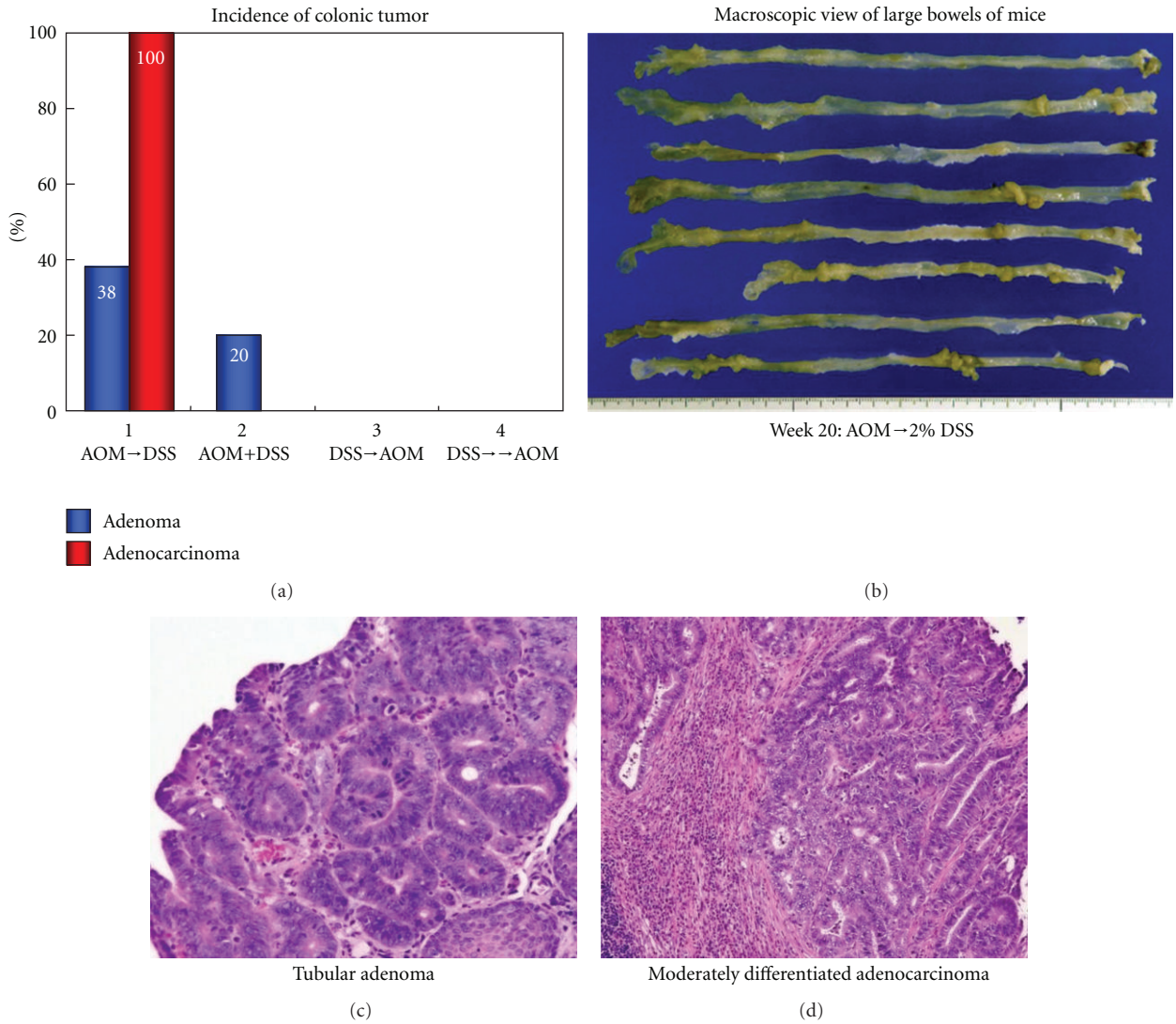



Figure 6: Macroscopic view, incidence, and histopathology of colonic tumors in the groups of mice that received four different treatment schedules of AOM and DSS.

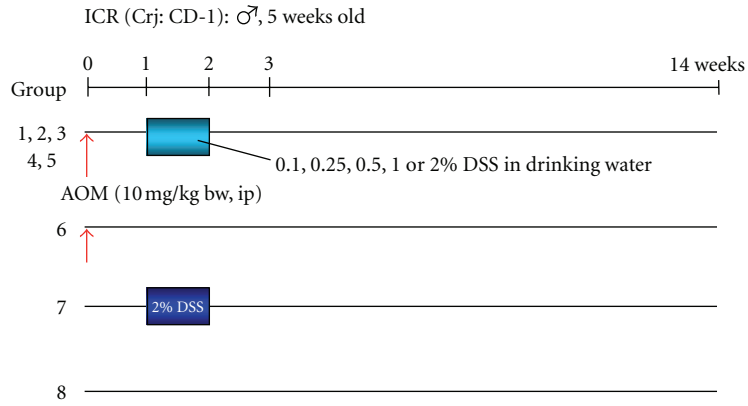



Figure 7: Experimental protocol for determining dose-response of DSS in mice initiated with AOM [21].

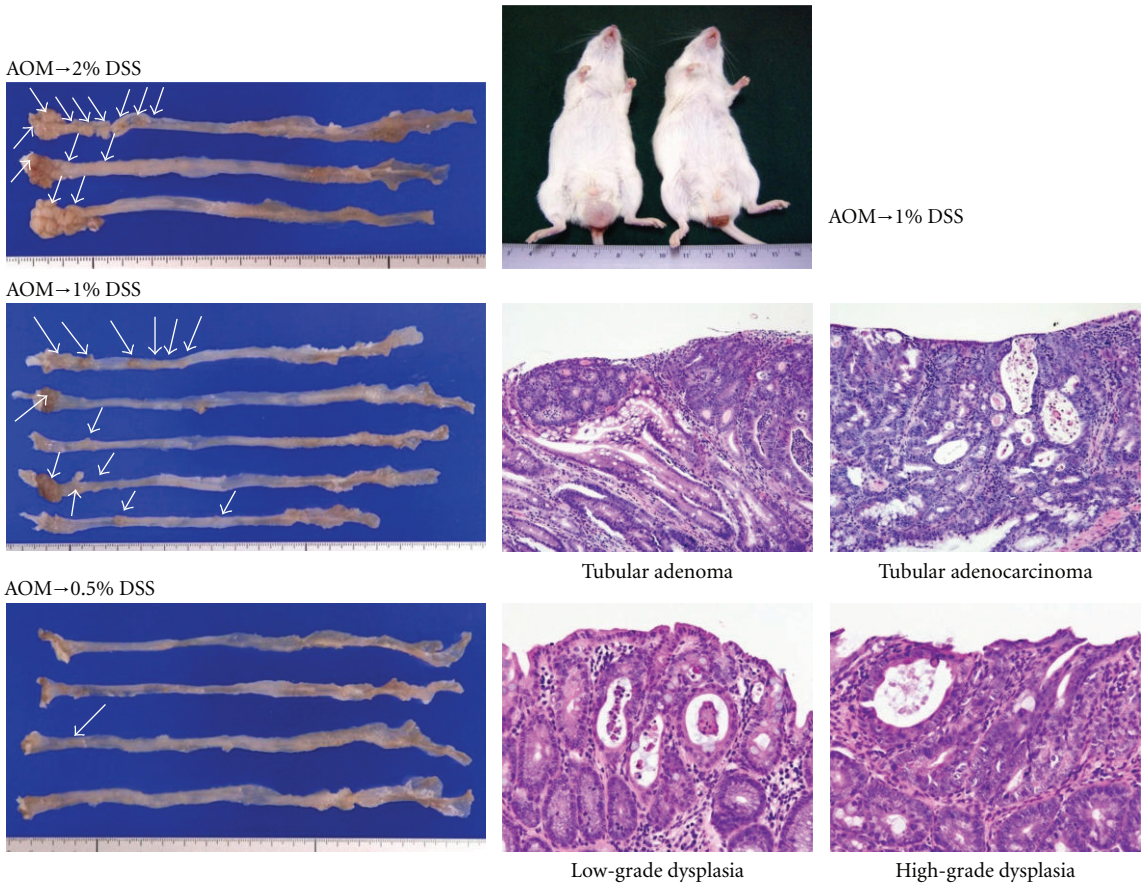



Figure 8: Macroscopic view and histopathology of colonic tumors developed in mice that received AOM and DSS (0.5%, 1%, or 2% DSS in drinking water).

Dose dependence of tumor-promotion activity of DSS after a single intraperitoneal administration of AOM (10 mg/kg body weight) was subsequently examined at five doses (0.1%, 0.25%, 0.5%, 1%, and 2%) of DSS (Figure 7) [21]. The findings indicated that tumor-promotion activity DSS was not observed at the concentration 0.25% or lower and only one tubular adenoma developed in a mouse that received AOM and 0.5% DSS. Colorectal tumors were developed in all mice by the treatment with 1% DSS and 2% DSS after AOM initiation and the number of colorectal adenocarcinoma was much greater in the group of mice treated with 2% DSS (Figure 8). The severity of colonic inflammation was determined by the histological inflammation score and immunohistochemical nitrotyrosine-positive reactivity. Both the inflammation score and nitrotyrosine-positive score in inflammatory cells that infiltrated colonic

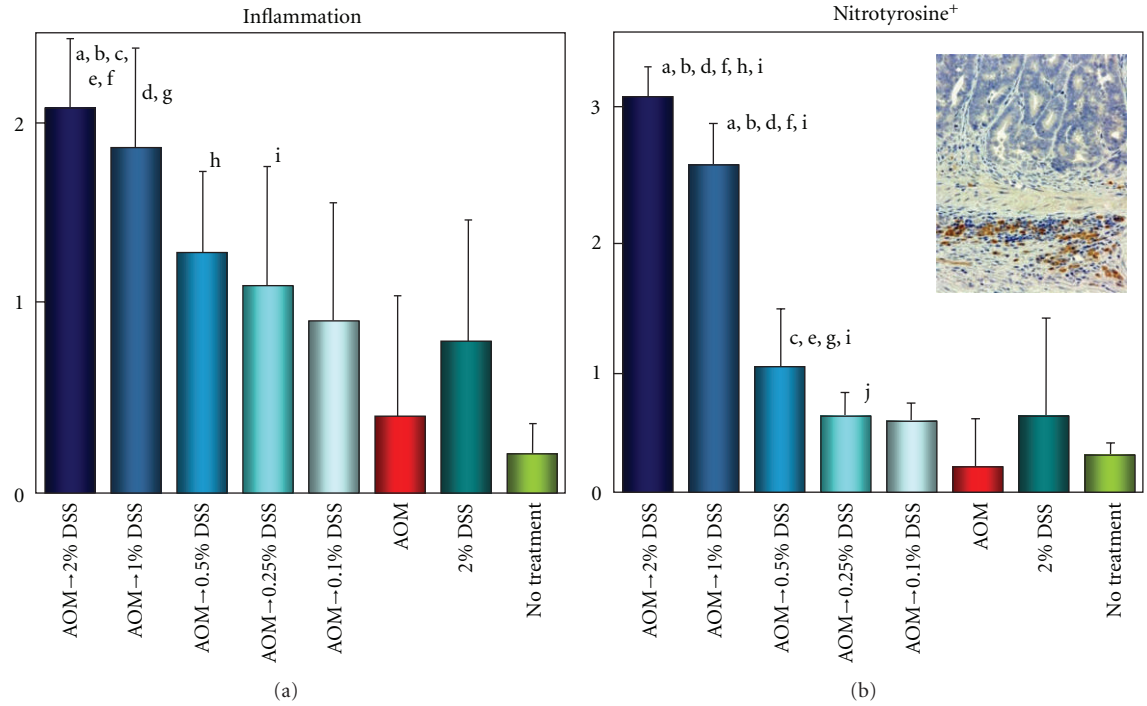



Figure 9: Inflammation and nitrotyrosine-positive scores in the colon of mice that received AOM and/or DSS (0.1%, 0.25%, 0.5%, 1%, or 2% DSS in drinking water). (a) Significantly different: a ($P < 0.05$), versus AOM → 0.5% DSS group; b ($P < 0.05$), versus AOM → 0.1% DSS group; c ($P < 0.01$) and d ($P < 0.05$), versus AOM alone group; e ($P < 0.05$), versus 2% DSS alone group; and f ($P < 0.001$), g ($P < 0.005$), h ($P < 0.01$), and i ($P < 0.05$), versus no treatment group. (b) Significantly different: a ($P < 0.001$), versus AOM → 0.5% DSS group; b ($P < 0.001$) and c ($P < 0.05$), versus AOM → 0.25% DSS group; d ($P < 0.001$) and e ($P < 0.01$), versus AOM → 0.1% DSS group; f ($P < 0.001$) and g ($P < 0.05$), versus AOM alone group; h ($P < 0.005$), versus 2% DSS alone group; and i ($P < 0.001$) and j ($P < 0.05$), versus no treatment group.

mucosa were higher in mice that received higher doses of DSS after AOM, suggesting that inflammation and nitrosation were involved in the tumor-promotion activity of DSS (Figure 9).

Time-course observation during AOM/DSS-induced mouse colorectal carcinogenesis was conducted to determine when colonic tumors occur in the inflamed colon of mice that received 2% DSS after the AOM initiation [22]. Male ICR mice were initiated with a single intraperitoneal injection of AOM (10 mg/kg body weight) and followed by one week administration with 2% DSS in drinking water. Our time-course observation revealed that colorectal adenoma and adenocarcinoma developed three and four weeks after AOM administration, respectively, and the numbers increased in a time-dependent manner during the follow-up period up to 14 weeks (Figure 10). Interesting finding of this study was that the high inflammation score and high nitrotyrosine-positive score lasted until five to six weeks after the cessation of DSS administration (Figure 11). Since mucosal ulcer caused by DSS administration was microscopically repaired at this point, persistence of the high nitrotyrosine-positive score, rather than the high inflammatory score, is intriguing as well as strong iNOS expression and weak PPAR$\gamma$ expression in the colonic mucosa at five and 10 weeks after the AOM administration (Figure 12).

Instead of AOM, experiments with DMH [23] or a heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [24] as an initiator (colonic carcinogene) and followed by DSS treatment showed similar results described previously (Figure 13). Histopathologically, adenocarcinoma induced by DMH/DSS showed severer atypia and more aggressive biological natures than that induced by AOM/DSS. As noticed in the cancers induced by AOM/DSS, the adenocarcinoma cells developed in the inflamed colon of mice that received DMH and DSS were positive for COX-2, iNOS, and $\beta$-catenin (Figure 14). Mutation patterns of the *$\beta$-catenin* gene were slightly among the adenocarcinomas that were induced by the different treatment regimens: AOM/DSS, codon 32–34, 37, and 41; DMH/DSS, codon 32, 34, 37, and 41; and PhIP/DSS, codon 32 and 34 (Figure 15). However, these mutations were restricted in the codon region (32–34, 37, 41, and 45) that played an important role in degradation of $\beta$-catenin protein.

There was a report of a difference in sensitivity of DSS-induced colitis among the species of mice [25]. To investigate whether the species differences influence inflammation-associated colorectal carcinogenesis, the sensitivity for different species of mice (Balb/c, C57BL/6N, C3H/HeN, and DBA/2N) were subjected to AOM/DSS-induced colorectal carcinogenesis [26]. The sensitivity to

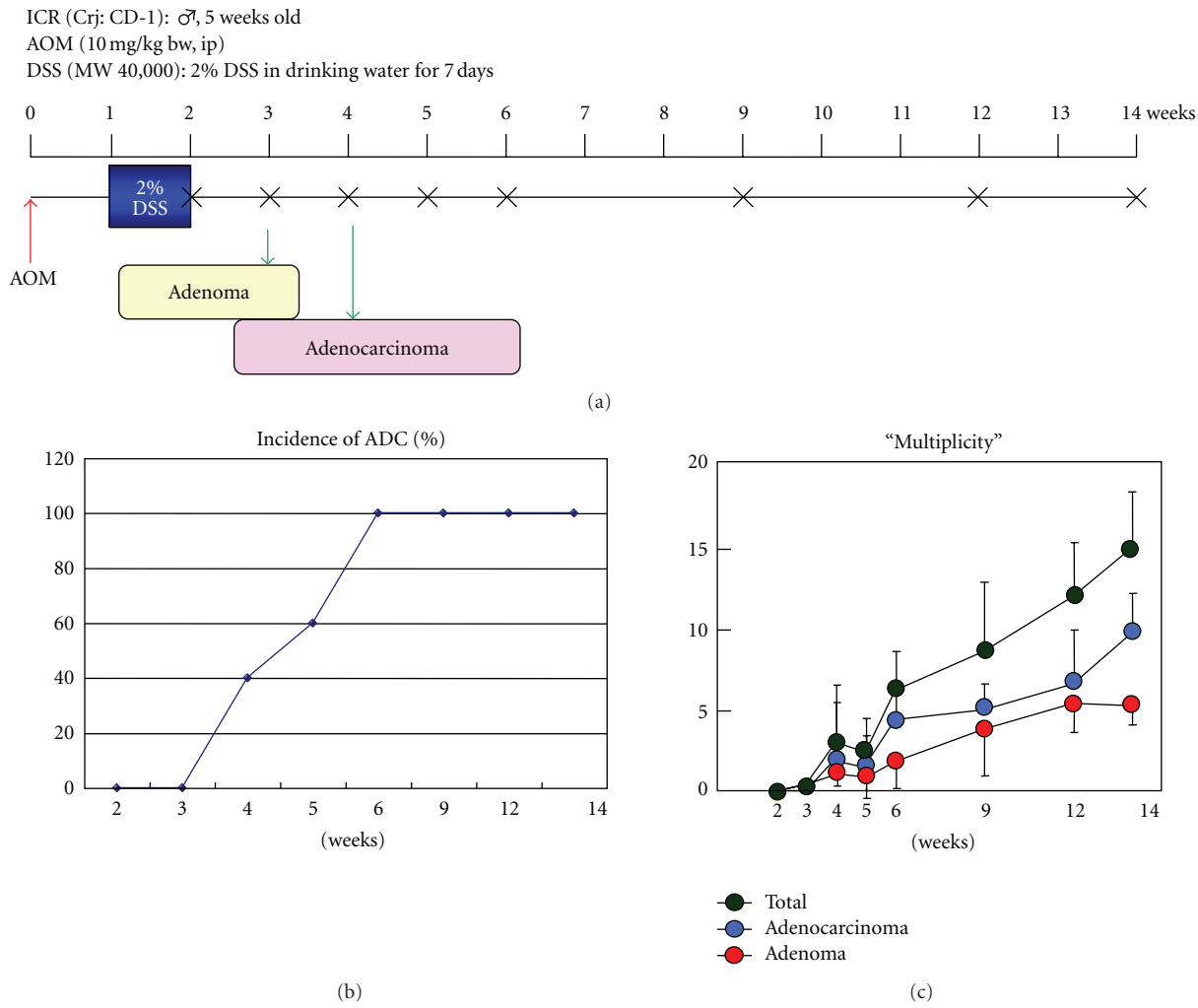



Figure 10: Experimental protocol of time-course observation of AOM/DSS-induce inflammation-associated colorectal carcinogenesis and tumor development (incidence and multiplicity) during the study [11].

Table 2: Animal models of colorectal carcinogenesis and inflammatory bowel disease. HCAs: heterocyclic amines.

| (1) Animal models of colorectal carcinogenesis |
|---|
| (i) *Carcinogen-induced animal models* |
| Azoxymethane (AOM) |
| 1,2-Dimethyl-hydrazine (DMH) |
| HCAs: 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) |
| 2-Amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (MeIQx) |
| (ii) *Mutant, transgenic, knockout animal models* |
| Min mouse and $APC^{\Delta 474}$ knockout mouse |
| (2) Animal models of inflammatory bowel disease |
| (i) *Chemically and polymer-induced models* |
| Trinitrobenzene sulfonic acid (TNBS): rat, mouse, rabbit |
| Dextran sulfate sodium (DSS): rat, mouse, hamster |
| Carrageenan: mouse, guinea pig, rabbit |
| (ii) *Microbial-induced models* |
| Cotton-top tamarins (*Saguinus oedipus*) |
| (iii) *Mutant mice* |
| IL-$2^{-/-}$, IL-$10^{-/-}$, TCR-$\alpha^{-/-}$, TCR-$\beta^{-/-}$ |

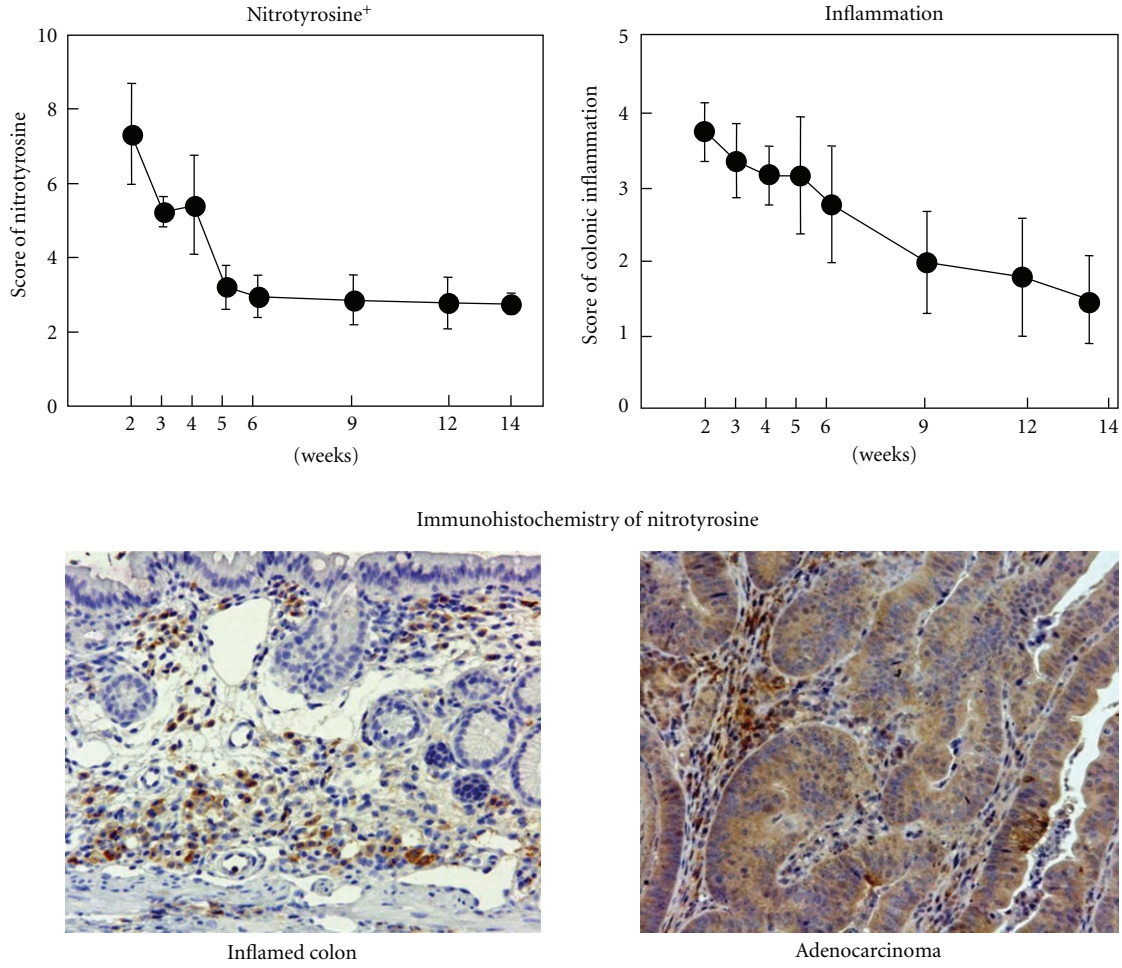



Figure 11: Scores of nitrotyrosine-positivity and inflammation in the inflamed colon and colonic tubular adenocarcinoma.

the AOM/DSS-induced colorectal carcinogenesis was as follows: Balb/c > C57BL/6N ≫ C3H/HeN = DBA/2N (Figure 16). The sensitivity was in relation to the nitrotyrosine-positive score estimated by immunohistochemical analysis, suggesting the importance of nitrotyrosine in the AOM/DSS-induced colorectal carcinogenesis [26].

In $Apc^{Min/+}$ mice, known as an animal model for familial adenomatous polyposis (FAP), multiple tumors (tubular adenomas) develop in the small intestine, instead of the large intestine in human FAP, and markedly few tumors develop in the large bowel. However, dysplastic crypts are observed in the colonic mucosa of $Apc^{Min/+}$ mice (Figure 17) [27, 28]. Therefore, DSS possibly enhances the growth of dysplastic crypts, and finally the lesions progress to adenocarcinomas. To investigate whether DSS-induced inflammation in the colonic mucosa would accelerate the growth of dysplastic crypts, $Apc^{Min/+}$ mice were given drinking water containing 2% DSS for one week without the initiation (carcinogen) treatment [29]. Surprisingly, multiple colorectal tumors, which were histopathologically tubular adenomas and adenocarcinomas, developed four weeks after the end of DSS treatment (Figure 18). Immunohistochemistry showed that the developed colorectal adenocarcinomas were positive against $\beta$-catenin, COX-2, iNOS, and p53 antibodies (Figure 19), suggesting that these factors were involved in the development of colorectal neoplasms in the $Apc^{Min/+}$ mice by the DSS treatment, in addition to oxidative stress and nitrosative stress. The findings suggested that DSS-induced inflammation in the large bowel of $Apc^{Min/+}$ mice exerts powerful tumor-promotion and/or progression effects on the growth of dysplastic crypts, which had already existed after the birth [27, 28].

Taken together, development of a mouse inflammation-associated colorectal carcinogenesis model was briefly described here, and the model was named as the TANAKA model. This model was possible to induce colorectal tumors in a short-term period in rats as well by similar treatment regimens (AOM/DSS and DMH/DSS) [30, 31]. It is anticipated that use of the TANAKA model will help advance the research on elucidation of the mechanisms of inflammation-associated colorectal carcinogenesis, inhibition of such carcinogenesis, and clarification of the mechanisms of the tumor-promotion ability of DSS. In particular, development of challenging research using Kyoto *Apc* Delta (KAD) rats in Kyoto University will give new insight in the pathogenesis of colorectal cancer development in the inflamed coon [32].

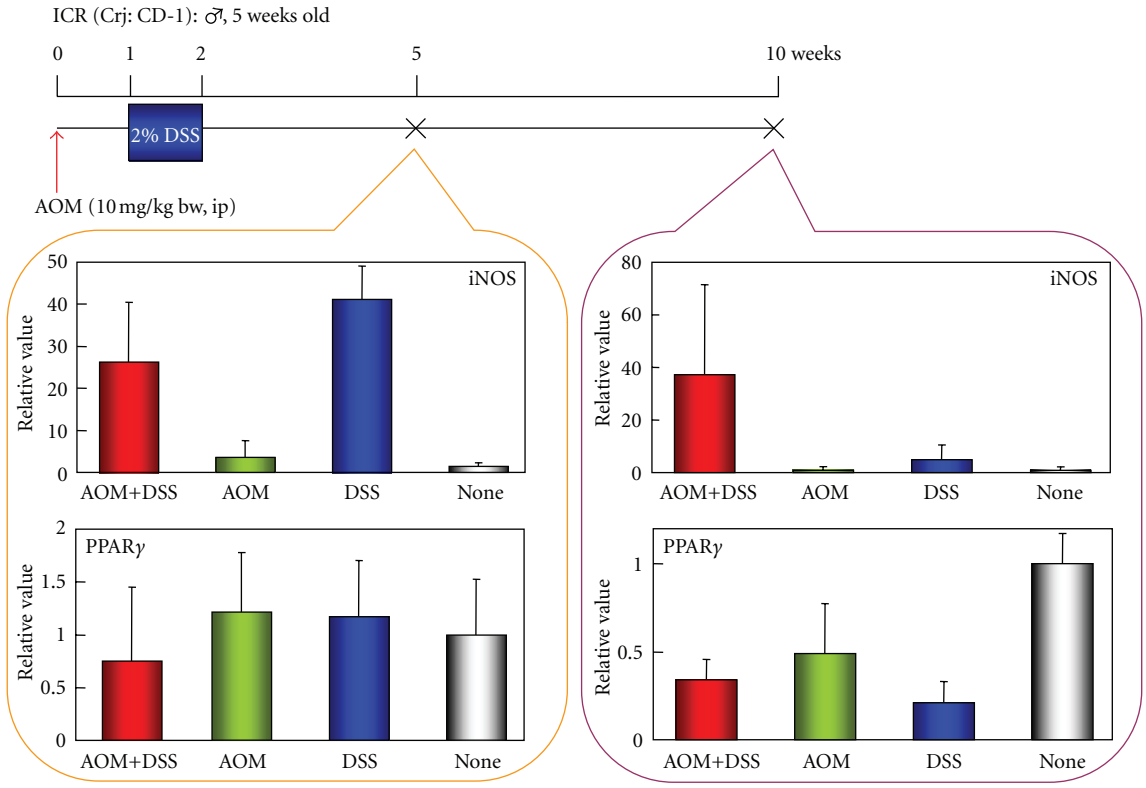
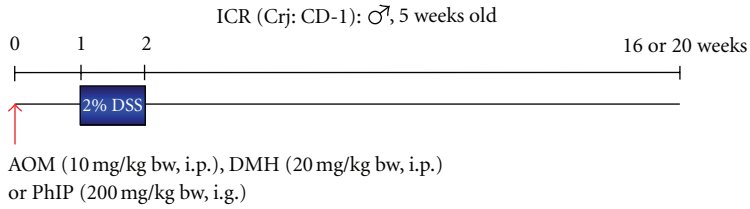



Figure 12: Real-time PCR analysis of iNOS and PPAR$\gamma$ in the colonic mucosa of mice that received AOM and DSS at weeks 6 and 10.




Incidence and multiplicity of colonic adenocarcinoma

| | AOM (20 weeks) | DMH (20 weeks) | PhIP (16 weeks) |
|---|---|---|---|
| Incidence | 100% | 100% | 56% |
| Multiplicity | 5.6 ± 2.4 | 5.8 ± 1.8 | 0.8 ± 1.0 |

Mutation in exon 3 of *β-catenin* in colonic adenocarcinoma

| | AOM (20 weeks) | DMH (20 weeks) | PhIP (16 weeks) |
|---|---|---|---|
| Frequency | 77% (10/13) | 91% (10/11) | 100% (7/7) |
| Mutated codon | 32,33,34 | 32,34,37,41 | 32,34 |

Figure 13: DSS is a powerful promoter in colon carcinogenesis in mice initiated with various colonic carcinogens, azoxymethane (AOM), 1,2-dimethylhydrazine (DMH), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [12, 21–24, 26].

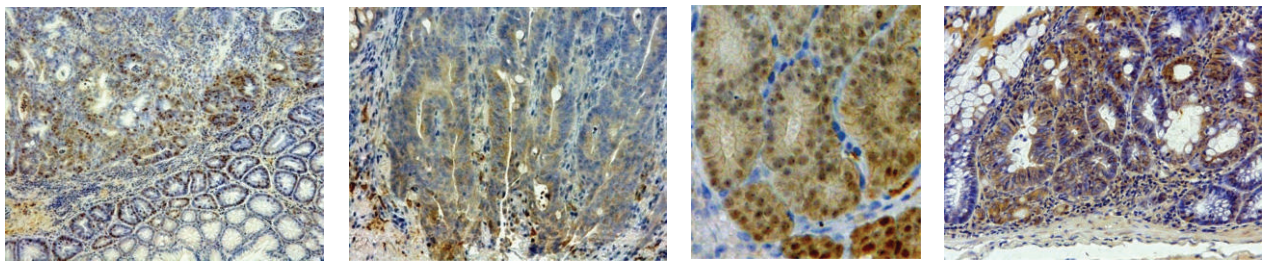



Figure 14: Immunohistochemistry of PCNA, $\beta$-catenin, COX-2, and iNOS in colonic adenocarcinomas of mice induced by AOM and DSS.

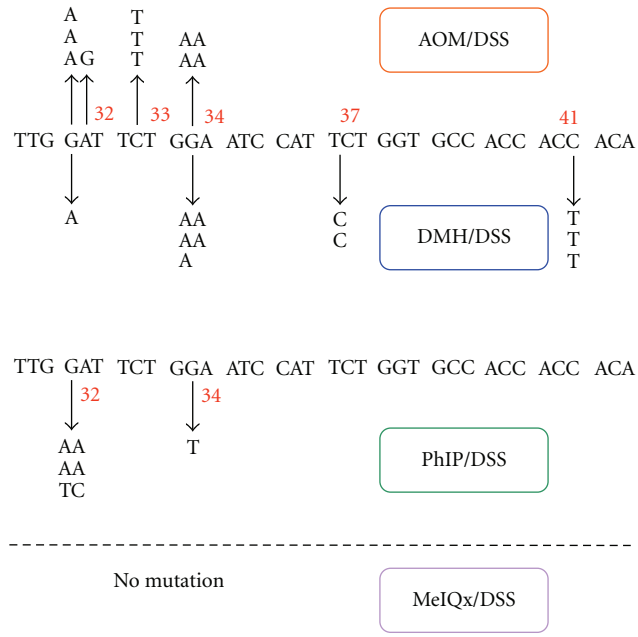



Figure 15: Mutations in the GSK-3$\beta$ phosphorylation consensus motif of the *$\beta$-catenin* gene in adenocarcinomas of mice induced by AOM/DSS, DMH/DSS, PhIP/DSS, and 2-amino-3,8-dimethylimidazo-[4,5-*f*]-quinoxaline (MeIQx)/DSS. PhIP and MeIQx are heterocyclic amines.

## 4. Exploration of Chemopreventive Agents Using an Inflammation-Associated Colorectal Carcinogenic Model and Elucidation of the Mechanisms

Studies on chemoprevention of inflammation-associated colorectal carcinogenesis by several natural and synthetic compounds against have been reported using the AOM/DSS-induced mouse and rat colorectal carcinogenesis models. Several are promising compounds and their clinical application is expected. Representative compounds are auraptene and nobiletin from citrus fruits [33], collinin [33], $\beta$-cyclodextrin inclusion compounds of auraptene and 4′-geranyloxyferulic acid [34], tricin [35], melatonin [30], ursodeoxycholic acid [36], COX-2 selective inhibitor nimesulide [37], iNOS selective inhibitors [38], PPAR ligands (troglitazone and bezafibrate) [37], and a lipophilic statin pitavastatin [39]. All these compounds have anti-inflammatory activity and are able to suppress the expression of COX-2, iNOS, and inflammatory cytokines.

## 5. Conclusions

Animal colorectal carcinogenesis models of our own making with the background of colitis mimicking human UC are introduced, and the exploration of chemopreventive compounds using these animal models is described. In addition, we confirmed upregulation of Wif1, Plat, Myc, and Plscr2 and downregulation of Pparbp, Tgfb3, and PPAR$\gamma$ by comprehensive gene expression analysis in the colonic

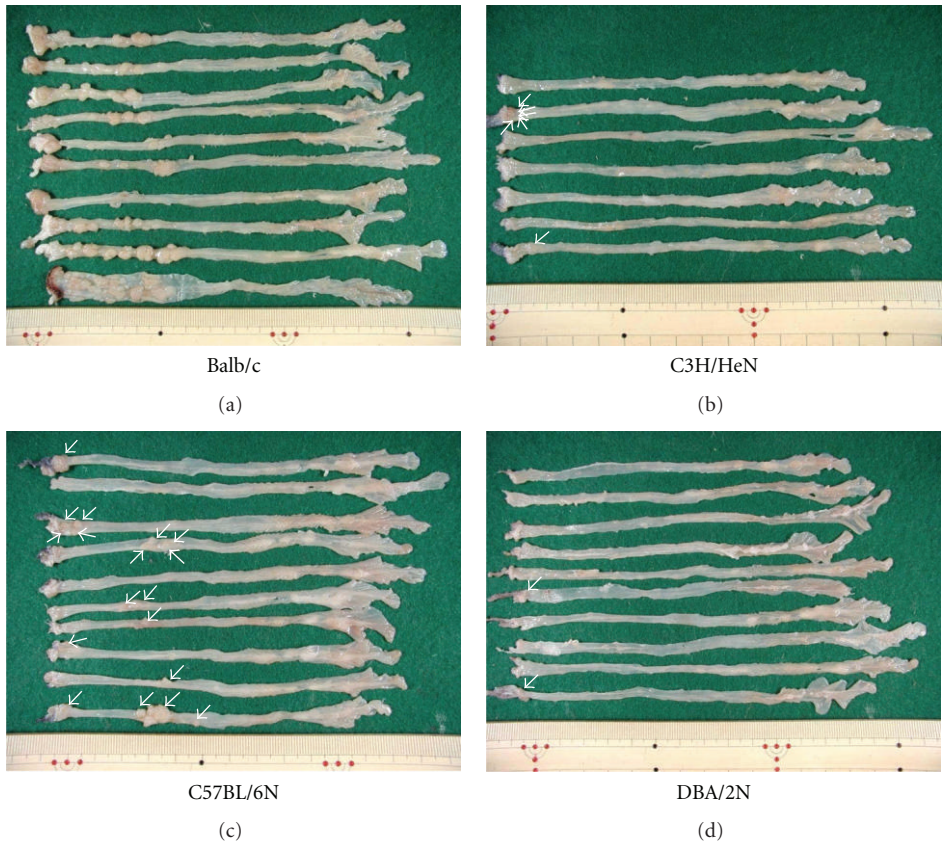



Figure 16: Macroscopic view of large bowel of four strains (Balb/c, C57BL/6N, C3H/HeN, and DBA/2N) of mice that received AOM and DSS.

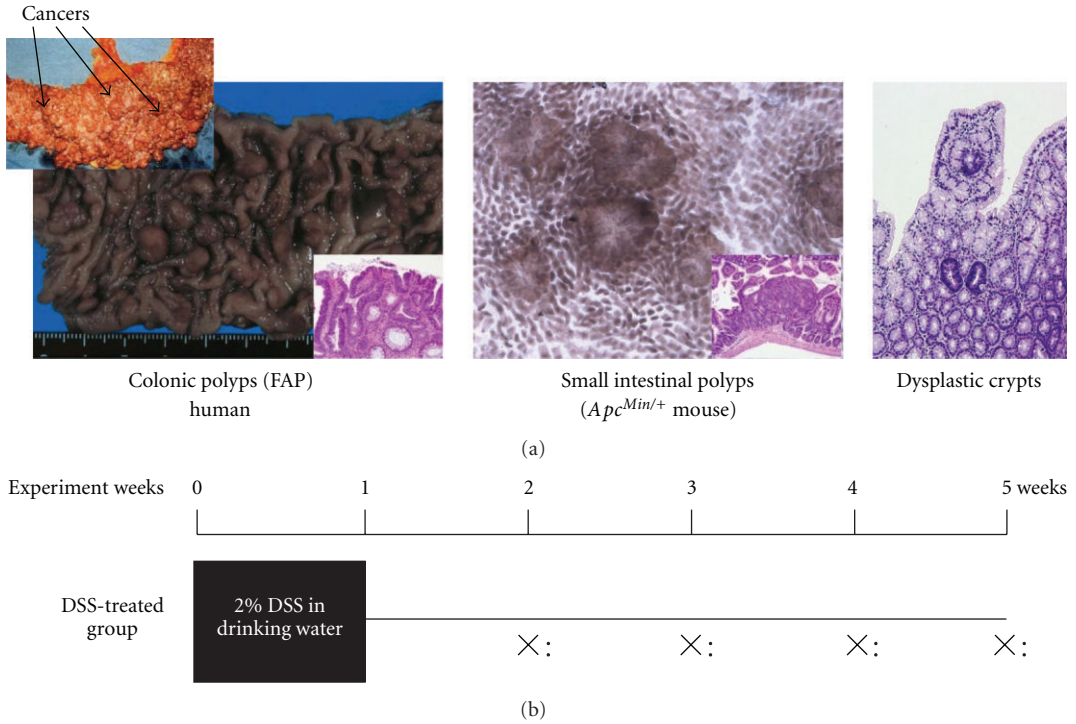



Figure 17: Colonic polyps in a familial adenomatous polyposis (FAP) patient and small intestinal polyps in an $APC^{Min/+}$ mouse (a). Experimental protocol for determining whether DSS promotes the growth of colonic dysplastic crypts in $APC^{Min/+}$ mice (b) [29].

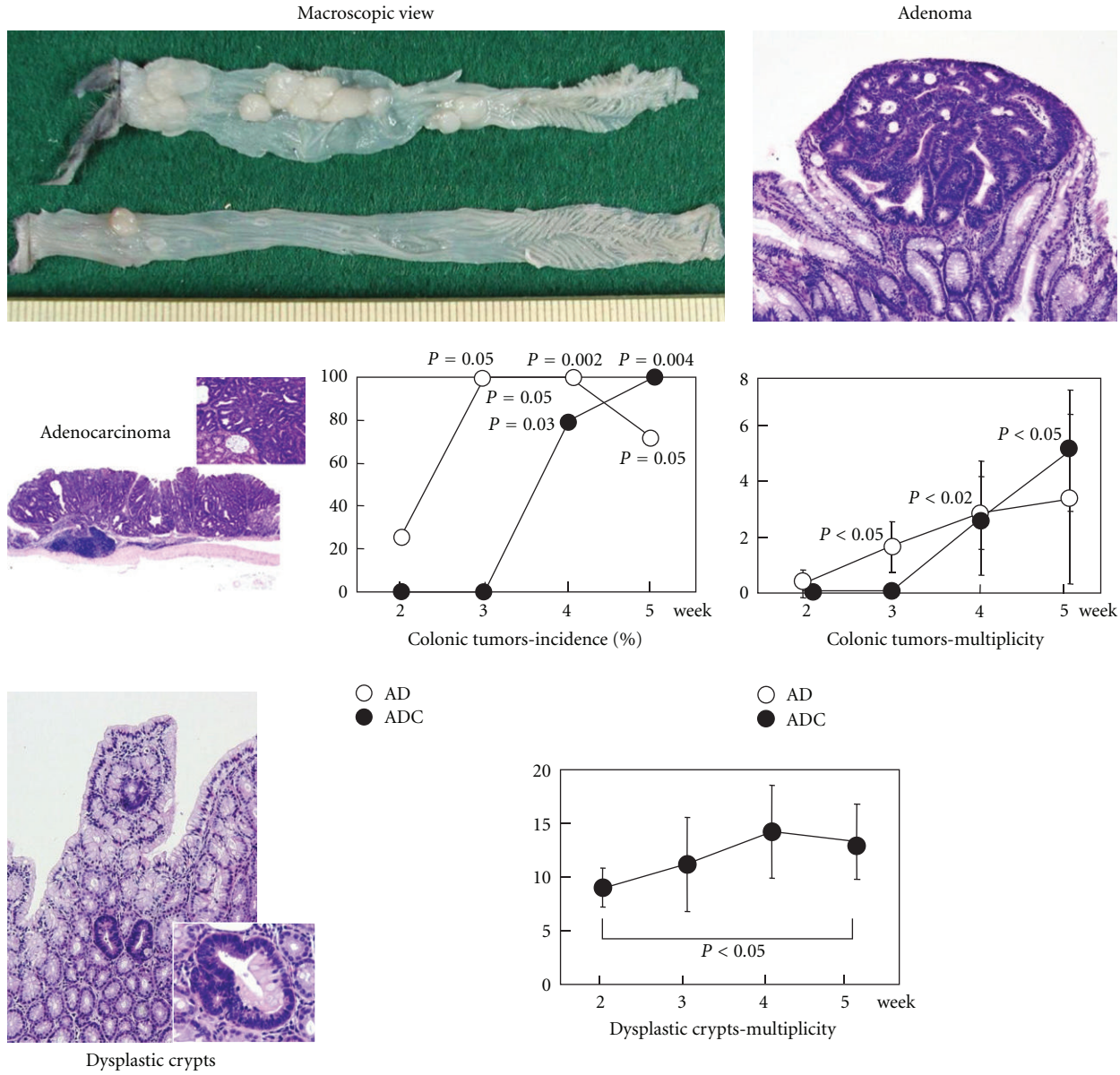
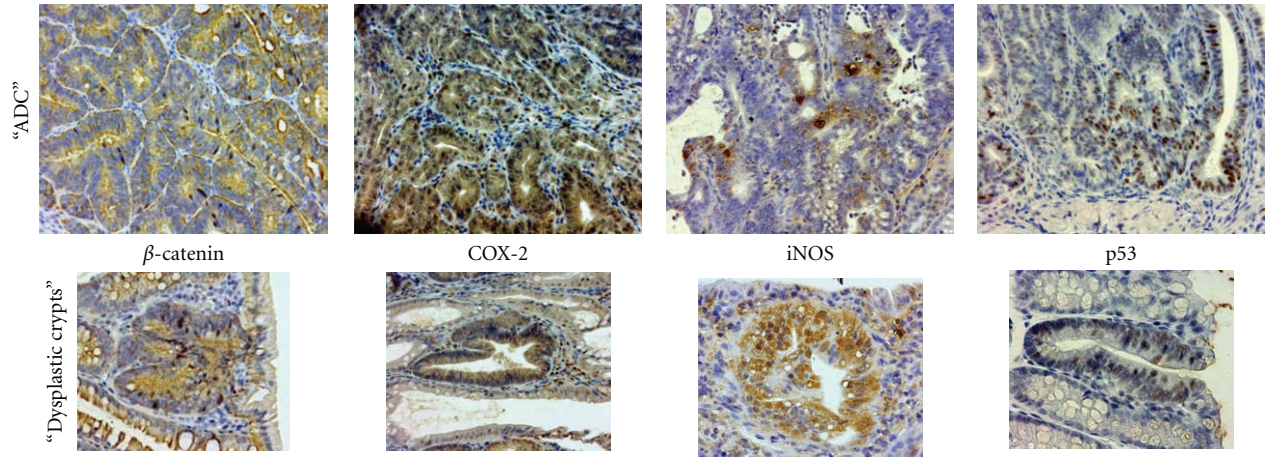



FIGURE 18: Macroscopic view and histopathology of colonic tumors and dysplastic crypts in $APC^{Min/+}$ mice that received 2% DSS for one week. Graphs show developments of these lesions during the study (up to 5 weeks).

mucosa of mice that received AOM and DSS [40]. Moreover, proteomics analysis demonstrated that beta-tropomyosin, tropomyosin 1 alpha isoform b, and S100 calcium binding protein A9 were upregulated, while Car1, selenium-binding protein 1, HMG-CoA synthase, thioredoxin 1, 1 Cys peroxiredoxin protein 2, Fcgbp protein, Cytochrome c oxidase subunit Va, and ETHE1 protein were downregulated [41]. Significance of expression of these genes and proteins in inflammation-associated colorectal carcinogenesis remains poorly understood and further detailed analysis is required. Since our recent study demonstrated that NF-$\kappa$B and Nrf2 were expressed in not only inflammatory cells but also cancer cells in the TANAKA (AOM/DSS) model [34], these molecules may be the targets for cancer chemoprevention against colorectal cancer in the inflamed colon. Moreover, modification of the protocol of the TANAKA model may help us to detect environmental carcinogens [42] and tumor-promoters [43] for the large bowel. Fortunately, the animal models introduced here have attracted attention of young researchers that are doing research on colorectal carcinogenesis, IBD, inflammation, and cancer. It is anticipated that use of these models will advance elucidation of the mechanisms (methylation and microRNA) of inflammation-associated colorectal carcinogenesis, exploration of its suppression and mechanisms, and clarification of the mechanisms of tumor-promotion activity of DSS.




| Treatment | *Apc* allelic loss | Gene mutation | |
|---|---|---|---|
| | | *β-catenin* | K-*ras* |
| DSS | 14/14 (100%) | 0/14 (0%) | 0/14 (0%) |
| Tap water | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |

Figure 19: Immunohistochemistry of β-catenin, COX-2, iNOS, and p53 in the colonic adenocarcinoma and dysplastic crypts developed in male $Apc^{Min/+}$ mice that received 2% DSS (upper panel). *Apc* allelic loss and gene mutations of *β-catenin* and K-*ras* in the colonic adenocarcinoma from male $Apc^{Min/+}$ mice (lower panel).

## Conflict of Interests

The author declare that he has no conflict of interests.

## Acknowledgments

This work was partly supported by a Grant-in-Aid for the 2nd and 3rd Terms Comprehensive 10-year Strategy for Cancer Control, Cancer Prevention, from the Ministry of Health and Welfare of Japan, a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan, and a Grant-in-Aid (no. 13671986 and no. 23501324) from the Ministry of Education, Science, Sports and Culture of Japan.

## References

[1] F. Balkwill and A. Mantovani, "Inflammation and cancer: back to Virchow?" *The Lancet*, vol. 357, no. 9255, pp. 539–545, 2001.

[2] B. J. Marshall and J. R. Warren, "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration," *The Lancet*, vol. 1, no. 8390, pp. 1311–1314, 1984.

[3] J. A. Eaden, K. R. Abrams, and J. F. Mayberry, "The risk of colorectal cancer in ulcerative colitis: a meta-analysis," *Gut*, vol. 48, no. 4, pp. 526–535, 2001.

[4] T. Tanaka, H. Kohno, M. Murakami, R. Shimada, and S. Kagami, "Colitis-related rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate (review)," *Oncology Reports*, vol. 7, no. 3, pp. 501–508, 2000.

[5] J. J. Y. Sung, J. Y. W. Lau, K. L. Goh et al., "Increasing incidence of colorectal cancer in Asia: implications for screening," *The Lancet Oncology*, vol. 6, no. 11, pp. 871–876, 2005.

[6] T. Tanaka, T. Oyama, and Y. Yasui, "Dietary supplements and colorectal cancer," *Current Topics in Nutraceutical Research*, vol. 6, no. 4, pp. 165–188, 2008.

[7] T. Tanaka and S. Sugie, "Inhibition of colon carcinogenesis by dietary non-nutritive compounds," *Journal of Toxicologic Pathology*, vol. 20, no. 4, pp. 215–235, 2007.

[8] Y. Yasui, M. Kim, T. Oyama, and T. Tanaka, "Colorectal carcinogensis and suppression of tumor development by inhibition of enzymes and molecular targets," *Current Enzyme Inhibition*, vol. 5, no. 1, pp. 1–26, 2009.

[9] T. Tanaka, "Colorectal carcinogenesis: review of human and experimental animal studies," *Journal of Carcinogenesis*, vol. 8, article 5, 2009.

[10] D. W. Rosenberg, C. Giardina, and T. Tanaka, "Mouse models for the study of colon carcinogenesis," *Carcinogenesis*, vol. 30, no. 2, pp. 183–196, 2009.

[11] M. Takahashi and K. Wakabayashi, "Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents," *Cancer Science*, vol. 95, no. 6, pp. 475–480, 2004.

[12] T. Tanaka, H. Kohno, R. Suzuki, Y. Yamada, S. Sugie, and H. Mori, "A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate," *Cancer Science*, vol. 94, no. 11, pp. 965–973, 2003.

[13] A. M. Lefebvre, I. Chen, P. Desreumaux et al., "Activation of the peroxisome proliferator-activated receptor γ promotes the

development of colon tumors in C57BL/6J-APC$^{Min/+}$ mice," *Nature Medicine*, vol. 4, no. 9, pp. 1053–1057, 1998.

[14] E. Saez, P. Tontonoz, M. C. Nelson et al., "Activators of the nuclear receptor PPAR$\gamma$ enhance colon polyp formation," *Nature Medicine*, vol. 4, no. 9, pp. 1058–1061, 1998.

[15] P. Sarraf, E. Mueller, D. Jones et al., "Differentiation and reversal of malignant changes in colon cancer through PPAR$\gamma$," *Nature Medicine*, vol. 4, no. 9, pp. 1046–1052, 1998.

[16] S. J. Alrawi, M. Schiff, R. E. Carroll et al., "Aberrant crypt foci," *Anticancer Research*, vol. 26, no. 1, pp. 107–119, 2006.

[17] R. P. Bird, "Role of aberrant crypt foci in understanding the pathogenesis of colon cancer," *Cancer Letters*, vol. 93, no. 1, pp. 55–71, 1995.

[18] A. K. Gupta, T. P. Pretlow, and R. E. Schoen, "Aberrant crypt foci: what we know and what we need to know," *Clinical Gastroenterology and Hepatology*, vol. 5, no. 5, pp. 526–533, 2007.

[19] T. Tanaka, H. Kohno, S. I. Yoshitani et al., "Ligands for peroxisome proliferator-activated receptors $\alpha$ and $\gamma$ inhibit chemically induced colitis and formation of aberrant crypt foci in rats," *Cancer Research*, vol. 61, no. 6, pp. 2424–2428, 2001.

[20] H. Mori, F. Ohbayashi, and I. Hirono, "Absence of genotoxicity of the carcinogenic sulfated polysaccharides carrageenan and dextran sulfate in mammalian DNA repair and bacterial mutagenicity assays," *Nutrition and Cancer*, vol. 6, no. 2, pp. 92–97, 1984.

[21] R. Suzuki, H. Kohno, S. Sugie, and T. Tanaka, "Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane," *Histology and Histopathology*, vol. 20, no. 2, pp. 483–492, 2005.

[22] R. Suzuki, H. Kohno, S. Sugie, and T. Tanaka, "Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate," *Cancer Science*, vol. 95, no. 9, pp. 721–727, 2004.

[23] H. Kohno, R. Suzuki, S. Sugie, and T. Tanaka, "$\beta$-catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate," *Cancer Science*, vol. 96, no. 2, pp. 69–76, 2005.

[24] T. Tanaka, R. Suzuki, H. Kohno, S. Sugie, M. Takahashi, and K. Wakabayashi, "Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess $\beta$-catenin gene mutations and increases immunoreactivity for $\beta$-catenin, cyclooxygenase-2 and inducible nitric oxide synthase," *Carcinogenesis*, vol. 26, no. 1, pp. 229–238, 2005.

[25] M. Mähler, I. J. Bristol, E. H. Leiter et al., "Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis," *American Journal of Physiology*, vol. 274, no. 3, pp. G544–G551, 1998.

[26] R. Suzuki, H. Kohno, S. Sugie, H. Nakagama, and T. Tanaka, "Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice," *Carcinogenesis*, vol. 27, no. 1, pp. 162–169, 2006.

[27] K. Hata, T. Tanaka, H. Kohno et al., "$\beta$-Catenin-accumulated crypts in the colonic mucosa of juvenile Apc$^{Min/+}$ mice," *Cancer Letters*, vol. 239, no. 1, pp. 123–128, 2006.

[28] Y. Yamada, K. Hata, Y. Hirose et al., "Microadenomatous lesions involving loss of Apc heterozygosity in the colon of adult Apc$^{Min/+}$ mice," *Cancer Research*, vol. 62, no. 22, pp. 6367–6370, 2002.

[29] T. Tanaka, H. Kohno, R. Suzuki et al., "Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc$^{Min/+}$ mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms," *International Journal of Cancer*, vol. 118, no. 1, pp. 25–34, 2006.

[30] T. Tanaka, Y. Yasui, M. Tanaka, T. Tanaka, T. Oyama, and K. W. Rahman, "Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats," *Chemico-Biological Interactions*, vol. 177, no. 2, pp. 128–136, 2009.

[31] N. Toyoda-Hokaiwado, Y. Yasui, M. Muramatsu et al., "Chemopreventive effects of silymarin against 1,2-dimethylhydrazine plus dextran sodium sulfate-induced inflammation-associated carcinogenicity and genotoxicity in the colon of gpt delta rats," *Carcinogenesis*, vol. 32, no. 10, pp. 1512–1517, 2011.

[32] K. Yoshimi, T. Tanaka, A. Takizawa et al., "Enhanced colitis-associated colon carcinogenesis in a novel Apc mutant rat," *Cancer Science*, vol. 100, no. 11, pp. 2022–2027, 2009.

[33] H. Kohno, R. Suzuki, M. Curini et al., "Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice," *International Journal of Cancer*, vol. 118, no. 12, pp. 2936–2942, 2006.

[34] T. Tanaka, M. B. de Azevedo, N. Durán et al., "Colorectal cancer chemoprevention by 2 $\beta$-cyclodextrin inclusion compounds of auraptene and 4′-geranyloxyferulic acid," *International Journal of Cancer*, vol. 126, no. 4, pp. 830–840, 2010.

[35] T. Oyama, Y. Yasui, S. Sugie, M. Koketsu, K. Watanabe, and T. Tanaka, "Dietary tricin suppresses inflammation-related colon carcinogenesis in male Crj: CD-1 mice," *Cancer Prevention Research*, vol. 2, no. 12, pp. 1031–1038, 2009.

[36] H. Kohno, R. Suzuki, Y. Yasui, S. Miyamoto, K. Wakabayashi, and T. Tanaka, "Ursodeoxycholic acid versus sulfasalazine in colitis-related colon carcinogenesis in mice," *Clinical Cancer Research*, vol. 13, no. 8, pp. 2519–2525, 2007.

[37] H. Kohno, R. Suzuki, S. Sugie, and T. Tanaka, "Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands," *BMC Cancer*, vol. 5, article 46, 2005.

[38] H. Kohno, M. Takahashi, Y. Yasui et al., "A specific inducible nitric oxide synthase inhibitor, ONO-1714 attenuates inflammation-related large bowel carcinogenesis in male Apc$^{Min/+}$ mice," *International Journal of Cancer*, vol. 121, no. 3, pp. 506–513, 2007.

[39] Y. Yasui, R. Suzuki, S. Miyamoto et al., "A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis," *International Journal of Cancer*, vol. 121, no. 10, pp. 2331–2339, 2007.

[40] R. Suzuki, S. Miyamoto, Y. Yasui, S. Sugie, and T. Tanaka, "Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate," *BMC Cancer*, vol. 7, article 84, 2007.

[41] Y. Yasui and T. Tanaka, "Protein expression analysis of inflammation-related colon carcinogenesis," *Journal of Carcinogenesis*, vol. 8, article 10, 2009.

[42] H. Kohno, Y. Totsuka, Y. Yasui et al., "Tumor-initiating potency of a novel heterocyclic amine, aminophenylnorharman in mouse colonic carcinogenesis model," *International Journal of Cancer*, vol. 121, no. 8, pp. 1659–1664, 2007.

[43] K. Hata, T. Tanaka, H. Kohno et al., "Lack of enhancing effects of degraded $\lambda$-carrageenan on the development of

$\beta$-catenin-accumulated crypts in male DBA/2J mice initiated with azoxymethane," *Cancer Letters*, vol. 238, no. 1, pp. 69–75, 2006.

[44] I. Hirono, I. Ueno, and S. Aiso, "Enhancing effect of dextran sulfate sodium on colorectal carcinogenesis by 1,2-dimethylhydrazine in rats," *Gann*, vol. 74, no. 4, pp. 493–496, 1983.

[45] I. Okayasu, M. Yamada, T. Mikami, T. Yoshida, J. Kanno, and T. Ohkusa, "Dysplasia and carcinoma development in a repeated dextran sulfate sodium-induced colitis model," *Journal of Gastroenterology and Hepatology*, vol. 17, no. 10, pp. 1078–1083, 2002.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 964720, 6 pages
doi:10.1155/2012/964720

*Review Article*

# Ultrasound of the Small Bowel in Crohn's Disease

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Received 16 September 2011; Revised 24 November 2011; Accepted 9 December 2011

Academic Editor: Christoph Gasche



Several radiological and endoscopic techniques are now available for the study of inflammatory bowel diseases. In everyday practice, the choice of the technique to be used depends upon its availability and a careful evaluation of diagnostic accuracy, clinical usefulness, safety, and cost. The recent development of innovative and noninvasive imaging techniques has led to a new and exciting area in the exploration of the gastrointestinal tract, especially in Crohn's disease patients by using ultrasound with oral or intravenous contrast.

## 1. Introduction

The diagnosis of Crohn's disease (CD) is based on clinical, endoscopic, radiological, and histological criteria. The main innovations in diagnostic technologies include the development of more sophisticated endoscopic and noninvasive imaging techniques with the aim of improving the identification of complications. Noninvasive tests for the diagnosis and followup of CD have gained increasing attention. Rapid and inexpensive noninvasive tests that are sensitive, specific, and simple to perform are necessary to prevent patient discomfort, delay in diagnosis, and unnecessary costs.

The use of transabdominal ultrasound (US) to evaluate gastrointestinal (GI) tract disorders is used primarily in the assessment of acute and chronic inflammatory conditions such as appendicitis, diverticulitis, ulcerative colitis, and CD [1]. Over the past few years, the technical evolution of ultrasound equipment, combined with the use of oral and intravenous contrast agents and the increased expertise of the operators, has led to a great enthusiasm for ultrasound assessment of the GI tract [2]. In chronic inflammatory conditions, mainly CD, these properties have not only been employed for diagnostic purposes but also been proposed for management and followup of the disease and its complications [3–7].

Bowel US has been largely promoted in continental Europe, where ultrasonography is carried out by a physician and is an integral part of the training curriculum for internal medicine, gastroenterology, surgery, and other fields. This technique is available in most European centers due to this training curriculum, whereas its use is less widespread in the United States.

## 2. CD Diagnosis

Bowel U.S. is now becoming the first-line imaging procedure in patients with suspected CD for early diagnosis of the disease [8]. Several studies have evaluated the significance of the U.S. detection of bowel wall thickness in the diagnosis of CD. Prospective studies, performed in unselected groups of patients, have shown that bowel U.S. may diagnose CD with a sensitivity ranging from 67–96% and specificity ranging from 79–100% [9–18]. Most of the results were obtained from studies that included patients with a previous diagnosis of CD but lacked of a control population; thus, it is difficult to achieve a comprehensive evaluation of sensitivity and specificity of the U.S. technique. These methodological problems were evaluated by Fraquelli and Conte [19]. In their meta-analysis, in which only five case-control and two cohort studies were ultimately considered from an initial 44 full-text studies identified, the impact of different cut-off values of bowel wall thickening (3 mm versus 4 mm) in determining the presence of CD was evaluated. The authors concluded that, using a cutoff level of 3 mm as normal, sensitivity and specificity were 88% and 93%, respectively.

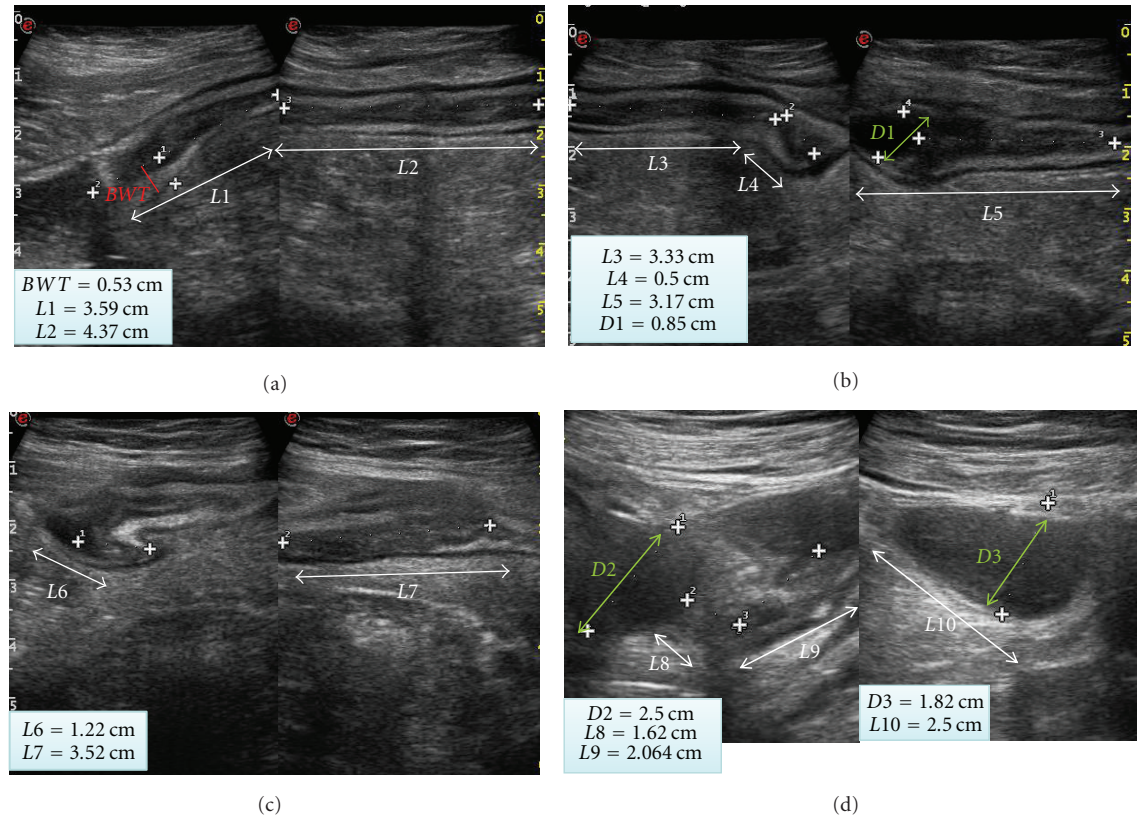


Figure 1: Small intestine contrast ultrasonography in a 20-year-old female with Crohn's disease. In each panel ((a)–(d)) white arrows indicate disease extent of the terminal ileum. The cumulative extent of the sonographic Crohn's disease lesion was 26 cm. In (a), on the left side, red arrow indicates bowel wall thickness (5.3 mm) of the terminal ileum and in (b) (on the left side) and (d) green (both right and left sides) arrows indicate lumen diameter (raging from 8 to 22 mm) at level of the terminal ileum.

In contrast, when a cutoff level of ≥4 mm was used, the sensitivity was 75% and specificity 97%. The meta-analysis conducted by Horsthuis et al. evaluated the relevance of US in the detection of IBD in comparison with other techniques [20]. No significant differences in diagnostic accuracy among the imaging techniques were observed. The authors concluded that because patients with IBD often needed frequent reevaluation of disease status, use of a diagnostic modality that does not involve the use of ionizing radiation is preferable [20]. However, the results also show that bowel US may, even in expert hands, be compounded by false-positive and false-negative findings. Thickening of the bowel walls is not specific for CD, also being present in infectious, neoplastic, and other inflammatory diseases [21]. Bowel US may also provide false-negative results, even in the hands of experienced ultrasonographers, for example, in obese patients or those with anorectal lesions only, or when the bowel disease is characterized by only superficial lesions, such as rare aphthous ulcers or mucosal erosions [22]. Interobserver agreement between sonographers with variable experience in bowel ultrasound has been reported in a few preliminary studies showing satisfactory results, but a learning curve for this technique is still lacking [14, 23]. This is probably one of the main reasons why bowel US is, in clinical practice, still regarded with skepticism by many clinicians and radiologists [6, 23].

The use of oral contrast agents such as iso-osmolar polyethylene glycol solution (PEG; at a volume ranging from 375–800 mL) during ultrasound assessment has been proposed to define CD lesions with improved accuracy [24–26] (Figure 1). Because of the small amount of fluid ingested (usually no more than 500 mL) and its palatability, this procedure has been reported to be wellaccepted and safe. None of the studies have reported significant side effects or major complaints during or immediately after PEG ingestion. The use of PEG appears to reduce intraobserver variability between sonographers and to increase sensitivity in defining disease extent, lesion site, and bowel complications of CD; thus, it has value in the early diagnosis and in the followup of CD [26, 27]. These findings suggest that small intestine contrast ultrasonography (SICUS) may be used as an alternative technique to invasive procedures to assess ileal lesions and monitor their progression over time.

## 3. Stenosis

Bowel US currently detects stenosis in 70–79% of unselected CD patients and in >90% of those with severe bowel stenoses needing surgery, with false-positive diagnoses limited to 7% [3, 4, 27, 28]. The use of PEG leads to a significantly greater accuracy of bowel ultrasound in detecting the presence and

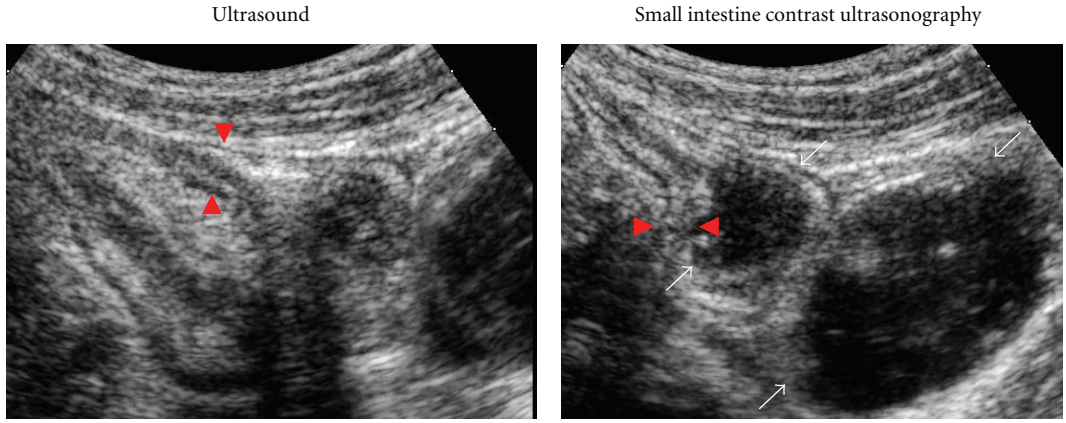


Figure 2: A 30-year-old male with stricturing Crohn's disease assessed by bowel ultrasound (without oral contrast) (a) and small intestine contrast ultrasonography (b). (a) shows Crohn's disease stenosis and prestenotic dilation, well defined in (b). Red arrowheads indicate bowel wall thickness in both panels, white arrows indicate prestenotic dilation in (b).

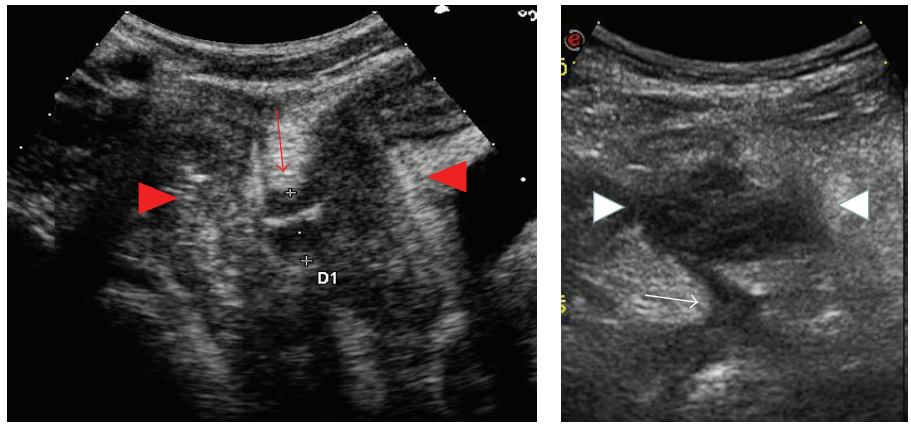


Figure 3: Bowel ultrasound in a 22-year-old female with ileocolonic Crohn's disease. In (a) red arrow indicates enteroenteric fistula (defined as a hypoechoic track with a hyperechoic content) between diseased ileal loops with bowel wall thickness (red arrowheads). In (b) a small abscess (white arrowheads identify a roundish anechoic lesions with irregular walls, presenting internal echoes) with fistula (white arrow) was identified in the same patient.

the number of stenoses. Bowel US with oral contrast detected at least one stenosis and at least two stenoses in >10% and >20% more patients, respectively, in comparison with bowel US without oral contrast agents, resulting in a sensitivity of approximately 90% for detection of a single stenosis and >75% for detection of multiple stenoses (Figures 2(a) and 2(b)) [26, 27].

## 4. Fistula

Two prospective studies have evaluated the role of US (without oral contrast) in determining the presence of internal fistulae (Figure 3(a)) using surgical and surgical-pathological findings as the reference standard. In one study, Gasche et al. reported a sensitivity of 87% and a specificity of 90% for bowel US in the detection of internal fistulae [4]. In the other prospective study, Maconi et al. determined the accuracy of bowel US and X-ray studies for detecting internal fistulae to be comparable with a sensitivity of 71.4% for US and 69.6% for X-ray, and specificity of 95.8% for both techniques. Maconi et al. showed also that the combination of these two techniques significantly improved preoperative diagnostic performance (sensitivity 97.4% and specificity 90%), with US being more accurate in detecting enteromesenteric fistulae while X-ray studies were superior in the diagnosis of enteroenteric fistulae [29]. In a recent study by Pallotta et al., SICUS identified fistulae in 27/28 patients and excluded it in 19/21 patients (96% sensitivity, 90.5% specificity) using surgery as gold standard [30]. In a recent systematic review, Panes and colleagues evaluated diagnostic accuracy of cross-sectional imaging techniques (US, CT and MR) for diagnosis of fistulas. These techniques showed higher accuracy than that of small bowel follow through (SBFT) [31]. CT and MR enterography

showed similar accuracy for the identification of extraenteric complications (sensitivity for both) [32, 33].

## 5. Abscess

Computed tomography (CT) and magnetic resonance imaging (MRI) are considered to be nonsurgical gold standard for the diagnosis of CD-related abscesses [31]. However, bowel US is also considered as a first-level procedure mainly because it is simple to use (Figure 3(b)). Four studies have prospectively assessed the accuracy of bowel US in the detection of intraabdominal abscesses, showing a mean sensitivity and specificity of 91.5% and 93%, respectively [3, 4, 29, 34]. In these studies, US showed a higher sensitivity in the detection of superficial intraperitoneal abscesses, whereas the diagnosis of deep pelvic or retroperitoneal abscesses was more difficult due to the presence of overlying bowel gas. Pallotta et al. showed that intraabdominal abscesses were correctly detected in 10/10 patients and excluded in 37/39 patients (100% sensitivity, 95% specificity, $k = 0.89$) by SICUS [30].

Contrast-enhanced ultrasound (CEUS) can be used to distinguish abscesses from inflammatory infiltrates [35].

## 6. Postoperative Recurrence

The sensitivity of bowel US in identifying the endoscopic recurrence after ileocolonic resection has been investigated in two studies showing 82% sensitivity [6, 36]. The use of PEG solution increased the sensitivity of ultrasound for assessing CD recurrence in patients under regular followup after ileocolonic resection. In our series, SICUS showed a high sensitivity (92.5%), positive predictive value (94%), and accuracy (87.5%) for detecting CD recurrence lesions using ileocolonoscopy as the gold standard [7].

## 7. CD Activity

The role of bowel US in the assessment of CD activity remains controversial. The degree of bowel wall thickening and extent of the thickened bowel wall on US (as an index of activity in CD) showed a significant but weak direct correlation between these features and clinical and biochemical parameters [22]. However, a statistically significant correlation was found between maximum bowel wall thickness and disease activity score in children and young adults [37].

Several studies have focused on the vascularity within the diseased bowel walls, assessed by power-Doppler US, as a quantitative method for determining CD activity. Three studies used Doppler US for detection of active disease, showing that wall thickness and vascularization pattern are useful for detection of active disease [38–40]. A recent study evaluating flow of the superior mesenteric artery confirmed previous observations regarding the correlation between disease activity and Doppler parameters [41–43].

The effectiveness of intravenous contrast agents in detection and assessing of bowel US activity of CD, despite some positive findings, remains controversial [40, 44–47]. The introduction of the microbubble contrast agents has enabled US to obtain information regarding the perfusion behavior of the organs and their diffuse or focal diseases. Enhancement in different wall layers can be evaluated and quantified in CD and correlates to clinical activity indices [35]. Migaleddu et al. reported in a prospective study that contrast-enhanced ultrasound (CEUS) showed 93.5% sensitivity, 93.7% specificity, and 93.6 overall accuracy in detecting inflammatory activity, calculated using the endoscopy/biopsy as gold standard. The linear correlation coefficient for CEUS versus Crohn's disease activity index (CDAI) was 0.74 ($P < 0.0001$) [40]. Ripolles et al. reported in a prospective study sensitivity and specificity of 96% and 73%, respectively, in the prediction of moderate or severe grade for inflammation at CEUS using endoscopy as gold standard [45]. More studies are needed to establish the exact role of CEUS in the imaging of GI pathology [35].

## 8. Conclusion

In recent years, several radiological and endoscopic techniques have been developed for the study of the small bowel. Bowel US has now become the first-line imaging procedure in patients with suspected CD for its early diagnosis. However, since the procedure is easy to use and offers good repeatability and accuracy, the most important indication of bowel US is currently in the followup of patients known to have CD. CEUS has been introduced as effective method in the quantitative and qualitative evaluation of CD inflammatory activity. In this context, these techniques may play a pivotal role in the early detection of intraabdominal complications, such as strictures, fistulae, and abscesses, and may be useful in the assessment of activity and in monitoring the course of disease during medical and postoperative followup, as a prognostic index of recurrence.

## Disclosures

The authors have no relevant financial interests to disclose.

## References

[1] P. J. Valette, M. Rioux, F. Pilleul, J. C. Saurin, P. Fouque, and L. Henry, "Ultrasonography of chronic inflammatory bowel diseases," *European Radiology*, vol. 11, no. 10, pp. 1859–1866, 2001.

[2] K. Schlottmann, W. Kratzer, and J. Scholmerich, "Doppler ultrasound and intravenous contrast agents in gastrointestinal tract disorders: current role and future implications," *European Journal of Gastroenterology and Hepatology*, vol. 17, no. 3, pp. 263–275, 2005.

[3] G. Maconi, S. Bollani, and G. B. Porro, "Ultrasonographic detection of intestinal complications in Crohn's disease," *Digestive Diseases and Sciences*, vol. 41, no. 8, pp. 1643–1648, 1996.

[4] C. Gasche, G. Moser, K. Turetschek, E. Schober, P. Moeschl, and G. Oberhuber, "Transabdominal bowel sonography for the detection of intestinal complications in Crohn's disease," *Gut*, vol. 44, no. 1, pp. 112–117, 1999.

[5] G. Maconi, L. Carsana, P. Fociani et al., "Small bowel stenosis in Crohn's disease: clinical, biochemical and ultrasonographic evaluation of histological features," *Alimentary Pharmacology and Therapeutics*, vol. 18, no. 7, pp. 749–756, 2003.

[6] A. Andreoli, P. Cerro, G. Falasco, L. A. Giglio, and C. Prantera, "Role of ultrasonography in the diagnosis of postsurgical recurrence of Crohn's disease," *American Journal of Gastroenterology*, vol. 93, no. 7, pp. 1117–1121, 1998.

[7] E. Calabrese, C. Petruzziello, S. Onali et al., "Severity of postoperative recurrence in Crohn's disease: correlation between endoscopic and sonographic findings," *Inflammatory Bowel Diseases*, vol. 15, no. 11, pp. 1635–1642, 2009.

[8] F. Parente, S. Greco, M. Molteni, A. Aderloni, G. Maconi, and G. B. Porro, "Modern imaging off Crohn's disease using bowel ultrasound," *Inflammatory Bowel Diseases*, vol. 10, no. 4, pp. 452–461, 2004.

[9] A. Sonnenberg, J. Erckenbrecht, P. Peter, and C. Niederau, "Detection of Crohn's disease by ultrasound," *Gastroenterology*, vol. 83, no. 2, pp. 430–434, 1982.

[10] A. Pera, T. Cammarota, E. Comino et al., "Ultrasonography in the detection of Crohn's disease and in the differential diagnosis of inflammatory bowel disease," *Digestion*, vol. 41, no. 3, pp. 180–184, 1988.

[11] J. Hata, K. Haruma, K. Suenaga et al., "Ultrasonographic assessment of inflammatory bowel disease," *American Journal of Gastroenterology*, vol. 87, no. 4, pp. 443–447, 1992.

[12] M. B. Sheridan, D. A. Nicholson, and D. F. Martin, "Transabdominal ultrasonography as the primary investigation in patients with suspected Crohn's disease or recurrence: a prospective study," *Clinical Radiology*, vol. 48, no. 6, pp. 402–404, 1993.

[13] T. Bozkurt, F. Richter, and G. Lux, "Ultrasonography as a primary diagnostic tool in patients with inflammatory disease and tumors of the small intestine and large bowel," *Journal of Clinical Ultrasound*, vol. 22, no. 2, pp. 85–91, 1994.

[14] J. Solvig, O. Ekberg, S. Lindgren, C. H. Floren, and P. Nilsson, "Ultrasound examination of the small bowel: comparison with enteroclysis in patients with Crohn disease," *Abdominal Imaging*, vol. 20, no. 4, pp. 323–326, 1995.

[15] S. Hollerbach, A. Geissler, H. Schiegl et al., "The accuracy of abdominal ultrasound in the assessment of bowel disorders," *Scandinavian Journal of Gastroenterology*, vol. 33, no. 11, pp. 1201–1208, 1998.

[16] M. Astegiano, F. Bresso, T. Cammarota et al., "Abdominal pain and bowel dysfunction: diagnostic role of intestinal ultrasound," *European Journal of Gastroenterology and Hepatology*, vol. 13, no. 8, pp. 927–931, 2001.

[17] F. Parente, S. Greco, M. Molteni et al., "Role of early ultrasound in detecting inflammatory intestinal disorders and identifying their anatomical location within the bowel," *Alimentary Pharmacology and Therapeutics*, vol. 18, no. 10, pp. 1009–1016, 2003.

[18] A. Rispo, M. Imbriaco, L. Celentano et al., "Noninvasive diagnosis of small bowel Crohn's disease: combined use of bowel sonography and Tc-99m-HMPAO leukocyte scintigraphy," *Inflammatory Bowel Diseases*, vol. 11, no. 4, pp. 376–382, 2005.

[19] M. Fraquelli and D. Conte, "The role of CT in coeliac disease. Methodology of the studies assessing diagnostic accuracy," *Digestive and Liver Disease*, vol. 37, no. 6, pp. 389–390, 2005.

[20] K. Horsthuis, S. Bipat, R. J. Bennink, and J. Stoker, "Inflammatory bowel disease diagnosed with US, MR, scintigraphy, and CT: meta-analysis of prospective studies," *Radiology*, vol. 247, no. 1, pp. 64–79, 2008.

[21] M. Truong, M. Atri, P. M. Bret et al., "Sonographic appearance of benign and malignant conditions of the colon," *American Journal of Roentgenology*, vol. 170, no. 6, pp. 1451–1455, 1998.

[22] G. Maconi, F. Parente, S. Bollani, B. Cesana, and G. Bianchi Porro, "Abdominal ultrasound in the assessment of extent and activity of Crohn's disease: clinical significance and implication of bowel wall thickening," *American Journal of Gastroenterology*, vol. 91, no. 8, pp. 1604–1609, 1996.

[23] M. Fraquelli, A. Sarno, C. Girelli et al., "Reproducibility of bowel ultrasonography in the evaluation of Crohn's disease," *Digestive and Liver Disease*, vol. 40, no. 11, pp. 860–866, 2008.

[24] F. Parente, G. Maconi, S. Bollani et al., "Bowel ultrasound in assessment of Crohn's disease and detection of related small bowel strictures: a prospective comparative study versus x ray and intraoperative findings," *Gut*, vol. 50, no. 4, pp. 490–495, 2002.

[25] N. Pallotta, E. Tomei, A. Viscido et al., "Small intestine contrast ultrasonography: an alternative to radiology in the assessment of small bowel disease," *Inflammatory Bowel Diseases*, vol. 11, no. 2, pp. 146–153, 2005.

[26] E. Calabrese, F. La Seta, A. Buccellato et al., "Crohn's disease: a comparative prospective study of transabdominal ultrasonography, small intestine contrast ultrasonography, and small bowel enema," *Inflammatory Bowel Diseases*, vol. 11, no. 2, pp. 139–145, 2005.

[27] F. Parente, S. Greco, M. Molteni et al., "Oral contrast enhanced bowel ultrasonography in the assessment of small intestine Crohn's disease. A prospective comparison with conventional ultrasound, x ray studies, and ileocolonoscopy," *Gut*, vol. 53, no. 11, pp. 1652–1657, 2004.

[28] A. Kohn, P. Cerro, G. Milite, E. De Angelis, and C. Prantera, "Prospective evaluation of transabdominal bowel sonography in the diagnosis of intestinal obstruction in Crohn's disease: comparison with plain abdominal film and small bowel enteroclysis," *Inflammatory Bowel Diseases*, vol. 5, no. 3, pp. 153–157, 1999.

[29] G. Maconi, G. M. Sampietro, F. Parente et al., "Contrast radiology, computed tomography and ultrasonography in detecting internal fistulas and intra-abdominal abscesses in Crohn's disease: a prospective comparative study," *American Journal of Gastroenterology*, vol. 98, no. 7, pp. 1545–1555, 2003.

[30] N. Pallotta, G. Vincoli, C. Montesani et al., "Small intestine contrast ultrasonography (SICUS) for the detection of small bowel complications in crohn's disease: a prospective comparative study versus intraoperative findings," *Inflammatory Bowel Diseases*, vol. 18, no. 1, pp. 74–84, 2012.

[31] J. Panes, R. Bouzas, M. Chaparro et al., "Systematic review: the use of ultrasonography, computed tomography and magnetic resonance imaging for the diagnosis, assessment of activity and abdominal complications of Crohn's disease," *Alimentary Pharmacology & Therapeutics*, vol. 34, no. 2, pp. 125–145, 2011.

[32] S. S. Lee, A. Y. Kim, S. K. Yang et al., "Crohn disease of the small bowel: comparison of CT enterography, MR enterography, and small-bowel follow-through as diagnostic techniques," *Radiology*, vol. 251, no. 3, pp. 751–761, 2009.

[33] G. Fiorino, C. Bonifacio, L. Peyrin-Biroulet et al., "Prospective comparison of computed tomography enterography and magnetic resonance enterography for assessment of disease activity and complications in ileocolonic Crohn's disease," *Inflammatory Bowel Diseases*, vol. 17, no. 5, pp. 1073–1080, 2011.

[34] H. Neye, D. Ensberg, P. Rauh et al., "Impact of high-resolution transabdominal ultrasound in the diagnosis of complications

of Crohn's disease," *Scandinavian Journal of Gastroenterology*, vol. 45, no. 6, pp. 690–695, 2010.

[35] F. Piscaglia, C. Nolsoe, and C. F. Dietrich, "The EFSUMB guidelines and recommendations on the clinical practice of contrast enhanced ultrasound (CEUS): update 2011 on non-hepatic applications," *Ultraschall in Medicin*, vol. 33, no. 1, pp. 33–59, 2012.

[36] G. DiCandio, F. Mosca, and A. Campatelli, "Sonographic detection of postsurgical recurrence of Crohn disease," *American Journal of Roentgenology*, vol. 146, no. 3, pp. 523–526, 1986.

[37] H. P. Haber, A. Busch, R. Ziebach, and M. Stern, "Bowel wall thickness measured by ultrasound as a marker of Crohn's disease activity in children," *The Lancet*, vol. 355, no. 9211, pp. 1239–1240, 2000.

[38] H. Neye, W. Voderholzer, S. Rickes, J. Weber, W. Wermke, and H. Lochs, "Evaluation of criteria for the activity of Crohn's disease by power Doppler sonography," *Digestive Diseases*, vol. 22, no. 1, pp. 67–72, 2004.

[39] M. J. Martínez, T. Ripollés, J. M. Paredes, E. Blanc, and L. Martí-Bonmatí, "Assessment of the extension and the inflammatory activity in Crohn's disease: comparison of ultrasound and MRI," *Abdominal Imaging*, vol. 34, no. 2, pp. 141–148, 2009.

[40] V. Migaleddu, A. M. Scanu, E. Quaia et al., "Contrast-enhanced ultrasonographic evaluation of inflammatory activity in Crohn's disease," *Gastroenterology*, vol. 137, no. 1, pp. 43–52, 2009.

[41] S. Karoui, K. Nouira, M. Serghini et al., "Assessment of activity of Crohn's disease by Doppler sonography of superior mesenteric artery flow," *Journal of Crohn's and Colitis*, vol. 4, no. 3, pp. 334–340, 2010.

[42] J. A. van Oostayen, M. N. Wasser, G. Griffioen, R. A. Van Hogezand, C. B. H. W. Lamers, and A. De Roos, "Diagnosis of Crohn's ileitis and monitoring of disease activity: value of Doppler ultrasound of superior mesenteric artery flow," *American Journal of Gastroenterology*, vol. 93, no. 1, pp. 88–91, 1998.

[43] G. Maconi, V. Imbesi, and G. Bianchi Porro, "Doppler ultrasound measurement of intestinal blood flow in inflammatory bowel disease," *Scandinavian Journal of Gastroenterology*, vol. 31, no. 6, pp. 590–593, 1996.

[44] C. Serra, G. Menozzi, A. M. Labate et al., "Ultrasound assessment of vascularization of the thickened terminal ileum wall in Crohn's disease patients using a low-mechanical index real-time scanning technique with a second generation ultrasound contrast agent," *European Journal of Radiology*, vol. 62, no. 1, pp. 114–121, 2007.

[45] T. Ripolles, M. J. Martínez, J. M. Paredes, E. Blanc, L. Flors, and F. Delgado, "Crohn disease: correlation of findings at contrast-enhanced US with severity at endoscopy," *Radiology*, vol. 253, no. 1, pp. 241–248, 2009.

[46] C. Girlich, E. M. Jung, E. Huber et al., "Comparison between preoperative quantitative assessment of bowel wall vascularization by contrast-enhanced ultrasound and operative macroscopic findings and results of histopathological scoring in crohn's disease," *Ultraschall in der Medizin*, vol. 32, no. 2, pp. 154–159, 2011.

[47] I. Sjekavica, V. Barbarić-Babić, Ž. Krznaric, M. Molnar, S. Čuković-Čavka, and R. Štern-Padovan, "Assessment of Crohn's disease activity by Doppler ultrasound of superior mesenteric artery and mural arteries in thickened bowel wall: cross-sectional study," *Croatian Medical Journal*, vol. 48, no. 6, pp. 822–830, 2007.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 412178, 11 pages
doi:10.1155/2012/412178

*Research Article*

# Pharmacological Evaluation of the SCID T Cell Transfer Model of Colitis: As a Model of Crohn's Disease

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Received 13 September 2011; Revised 21 October 2011; Accepted 5 November 2011

Academic Editor: Christoph Gasche



Animal models are important tools in the development of new drug candidates against the inflammatory bowel diseases (IBDs) Crohn's disease and ulcerative colitis. In order to increase the translational value of these models, it is important to increase knowledge relating to standard drugs. Using the SCID adoptive transfer colitis model, we have evaluated the effect of currently used IBD drugs and IBD drug candidates, that is, anti-TNF-$\alpha$, TNFR-Fc, anti-IL-12p40, anti-IL-6, CTLA4-Ig, anti-$\alpha 4\beta 7$ integrin, enrofloxacin/metronidazole, and cyclosporine. We found that anti-TNF-$\alpha$, antibiotics, anti-IL-12p40, anti-$\alpha 4\beta 7$ integrin, CTLA4-Ig, and anti-IL-6 effectively prevented onset of colitis, whereas TNFR-Fc and cyclosporine did not. In intervention studies, antibiotics, anti-IL-12p40, and CTLA4-Ig induced remission, whereas the other compounds did not. The data suggest that the adoptive transfer model and the inflammatory bowel diseases have some main inflammatory pathways in common. The finding that some well-established IBD therapeutics do not have any effect in the model highlights important differences between the experimental model and the human disease.

## 1. Introduction

The two inflammatory bowel diseases (IBDs) ulcerative colitis (UC) and Crohn's disease (CD) affect more than 3.6 million people in the Western world, resulting in a marked decrease in the patients' quality of life [1, 2]. The aetiology is poorly understood, but it has become clear that genetic, microbial, and environmental factors all play a role [3]. A massive effort is taking place to develop new and better therapeutics, and the development of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) antagonists has ameliorated the disease in a large proportion of especially CD patients [4]. However, about one third of the CD patients do not respond to anti-TNF-$\alpha$ treatment and among the primary responders, about one third loose response or become intolerant to the treatment [5], thus leaving many IBD patients with inadequate therapeutic options.

New IBD drugs and drug candidates include anti-interleukin (IL)-12/-23 (e.g., ustekinumab, briakinumab), cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4-Ig, abatacept), anti-IL-6R (tocilizumab), anti-interferon $\gamma$ ((IFN-$\gamma$), fontolizumab), anti-$\alpha 4\beta 7$ (vedolizumab), anti-$\alpha 4$ integrin (natalizumab), anti-IL-2-R$\alpha$ (daclizumab, basiliximab), antigranulocyte macrophage colony-stimulating factor (anti-GM-CSF, sagramostim), anti-intercellular adhesion molecule 1 (anti-ICAM-1, alicaforsen), rIL-18 binding protein (tadekinig-$\alpha$), IP-10/CXCL10 (MDX-1100), anti-CD3 (visilizumab), and anti-CD40L (TNX 100) [6, 7]. These compounds aim at targeting specific immunological mechanisms like cellular adhesion (anti-$\alpha 4\beta 7$, anti-ICAM-1) and costimulation (CTLA4-Ig, anti-CD40L), key cytokines (anti-IL-12/-23, anti-IL-6R, anti-IFN-$\gamma$) or cells (anti-IL-2R$\alpha$/CD25, anti-CD3), or have specific immuno-stimulatory (GM-CSF) or -inhibitory (rIL-10) effects.

Animal models are essential for dissecting the role of the pathological mechanisms in IBD as well as for assessing the therapeutic effect of intervening with these pathways [8]. To what extent the data from animal models can be translated into the clinic differs among the various models depending, for example, on the model's etiopathogenesis and main drivers of disease. There is no single model which adequately mimics either UC or CD, and to be able to translate findings from a model to the human disease, it is important to know the model's central pathological mechanisms and immunological pathways.

Adoptive transfer of a subset of $CD4^+$ T cells to syngeneic SCID or Rag-knock-out mice, results in the development of a chronic, progressive colitis and wasting disease as first described by Morrissey et al. and Powrie et al. [9, 10]. The colitis symptoms share several features with both CD and UC (e.g., chronic, progressive disease with diarrhoea and weight loss, heavily inflamed colon—occasionally transmural damage, loss of mucus from goblet cells, Th1/Th17 dominated cytokine profile as found in CD (IFN-$\gamma$, TNF-$\alpha$, and IL-23). The model has been extensively used for studying the immunologic background for the disease as well as testing new IBD drug candidates [11–14]. We have previously described in detail the development of colitis following adoptive transfer of $CD4^+CD25^-$ T cells [15]. Briefly, in our hands, the adoptively transferred cells expand rapidly and the mice begin to develop colitis within the first two weeks. At week three, the disease is normally fully developed with weight loss, loose stools, increased white blood cell (WBC) count, and a both thickened and shortened colon. The disease progresses rapidly and by week 5 most mice have developed severe colitis requiring a termination of the study. This synchronized and predictable development of colitis makes it possible to conduct both prevention and intervention studies.

The aim of this study was to analyze the model with respect to its usefulness in efficacy studies of new IBD drug candidates by evaluating the effect of known and potential IBD therapeutics in the model. We have decided to study a number of compounds, which each has a specific inhibitory effect on a central proinflammatory pathway. In addition, we have included some established IBD therapies, suggested to ameliorate IBD by a broad spectrum of mechanisms.

## 2. Materials and Methods

*2.1. Materials.* Human CTLA4-Ig (abatacept, Orencia, Bristol-Myers Squibb), human tumor necrosis factor receptor Fc (TNFR-Fc) (etanercept, Enbrel, Wyeth), enrofloxacin (Baytril, Bayer, equivalent to ciprofloxacin), metronidazole (Flagyl, Sanofi-Aventis), cyclosporine (Sandimmun, Novartis). All surrogate antibodies, that is, anti-TNF-$\alpha$ (clone XT3.11, rat IgG1), anti-IL-12p40 (clone C17.8, rat IgG2a), anti-IL-6 (MP5-20F3, rat IgG1), anti-$\alpha4\beta7$ (clone DATK32, rat IgG2a), and isotype controls (cIg) (rat IgG2a clone 2A, rat IgG1 clone HRPN and human IgG1-Fc) were from BioXCell, West Lebanon, New Hampshire, USA.

Dynabeads, Mouse CD4 (L3T4), and DETACHaBEAD Mouse were from Dynal, Oslo, Norway, and CD25 MicroBead kit from Miltenyi Biotech, Bergisch Gladbach, Germany. The antibodies used for FACS analysis were PerCP-conjugated anti-CD4 (L3T4) from BD Pharmingen and FITC-conjugated anti-CD45.2 (104) from eBiosciences, CA, USA.

*2.2. Mice.* C.B-Igh-1b/IcrTac-Prkdcscid (C.B-17 SCID) and BALB/cAnNTac female mice (8–10 weeks) bred under SPF conditions (M&B Taconic, Denmark) were housed at Novo Nordisk A/S. Pathology screening was conducted according to FELASA guidelines. The animal studies were approved by the Danish Animal Experimentation Inspectorate.

*2.3. Induction of Colitis.* For induction of colitis, $CD4^+$ $CD25^-$ T cells were adoptively transferred from MHC-compatible Balb/c mice to C.B-17 SCID recipients as described previously in [15]. In brief, Balb/c splenocytes were positively selected for $CD4^+$ T cells using Dynabeads and DETACHaBEAD and depleted of $CD4^+CD25^+$ cells using the CD25 MicroBead kit. The purity of the cells was always analyzed by flow cytometry before reconstitution (>98% of the $CD4^+$ cells were $CD25^-$). The recipients were reconstituted with 300,000 cells by i.p. injection. Peripheral blood from all mice was subjected to flow cytometric analysis 2 or 3 weeks after transfer, and only mice with $CD4^+$ T cells (indicating successful transplantation of cells) were included in the study.

*2.4. Experimental Setup.* The drugs tested, as well as the doses and dosing regimens are described in Table 1. For prevention studies, the mice were treated from the day they were adoptively transferred with $CD4^+CD25^-$ T cells and until sacrifice when the disease was fully developed (three or four weeks after transfer, Figure 1). For the intervention studies, the treatment was initiated at week three after adoptive transfer, when the $CD4^+$ T cells had expanded and caused colitis in the recipients. The treatment was continued for two weeks until sacrifice at week five. The control groups for the biologics (except for TNFR-Fc) were treated with the relevant control immunoglobulin (cIg), that is, rat IgG1 for anti-TNF-$\alpha$ and anti-IL-6, rat IgG2a for anti-IL-12p40 and anti-$\alpha4\beta7$, and human IgG1-Fc for CTLA4-Ig. The vehicle groups for cyclosporine and antibiotics received sterile $H_2O$ (Table 1). In the study with antibiotics, we also included a group, which was not reconstituted but received treatment, since we suspected that disturbance of the gut microflora in itself could have a marked effect on the measured disease parameters. We used the same doses and dosing frequencies for the various compounds in the prevention and intervention studies (Table 1). The selection of doses and dosing frequencies were based on either literature describing efficacious treatment in various colitis models or based on our experience with efficacious treatment in the collagen induced arthritis model [16–23].

*2.5. Monitoring of Disease.* Body weight was determined three times weekly, and mice were sacrificed if they lost more than 20% of their initial body weight. Fecal consistency was

Table 1: Study design—administration of compounds.

| Compound | Dose (mg/kg) | cIg/Vehicle | Mice per group[1] | Dose/wk | Route |
|---|---|---|---|---|---|
| Rat anti-mouse TNF-$\alpha$ | 25 | rat IgG1 | $15^{P}/10^{I,2}$ | 2 | i.p. |
| Human TNFR-Fc (IgG1) | 5–50 | NaCl | $10^{P+I}$ | 3 | i.p. |
| Rat anti-mouse IL-12p40 | 25 | rat IgG2a | $10^{P+I}$ | 3 | i.p. |
| Rat anti-mouse-IL-6 | 25 | rat IgG1 | $10^{P+I}$ | 3 | i.p. |
| Human CTLA4-Ig (IgG1) | 10 | hIgG1-Fc[3] | $10^{P+I}$ | 3 | i.p. |
| Rat anti-mouse-$\alpha 4\beta 7$ | 25 | rat IgG2a | $10^{P+I}$ | 3 | i.p. |
| Enro/metro[4] | 350/875 | $H_2O$ | $9^{P}/10^{I}$ | daily | p.o. |
| Cyclosporine | 25 | $H_2O$ | $10^{P}$ | daily | p.o. |

[1] Five to ten unreconstituted mice were included in addition to the compound and the control group.
[2] P: prevention, I: intervention.
[3] human IgG1-Fc.
[4] Treatment with enrofloxacin and metronidazole in the drinking water was initiated one week prior to transfer to let the mice adjust to the taste. Although we in pilot studies had identified a useful sugar mixture to mask the taste of metronidazole, the mice refused to drink and lost weight prior to adoptive transfer in the prevention study. Metronidazole was subsequently given orally by gavage once daily (this method was then also used for the intervention study).

evaluated before the start of treatment and at the termination of the study using a semiquantitative score (normal stool = 0; slightly soft = 1; soft but formed = 2; not formed = 3; liquid stools or no feces in colon at sacrifice = 4) as previously described [15]. The number of WBC per liter was analyzed in samples (20 $\mu$L) of EDTA-stabilized peripheral whole blood, using a Medonic CA 620 (Boule Nordic, Denmark) blood analysis apparatus according to the manufacturer's instructions.

*2.6. Postmortem Analysis.* Prior to sacrifice, the mice were anesthetized and blood from the periorbital venous plexus was collected in EDTA-containing tubes. After sacrifice, the colon was excised, rinsed gently with saline, and the weight and length recorded. The colonic weight-to-length ratio (W : L) was previously shown to correlate strongly with the clinical and histological severity of disease [15]. The colon was opened longitudinally, mounted on a plastic plate, and fixed overnight in 4% paraformaldehyde.

*2.7. Histology.* Longitudinal segments of tissue representing essentially the entire length of the transverse and distal colon (where the inflammation is mainly located) were embedded in paraffin. A section (7 $\mu$m) of the transverse and the distal colon from each animal was stained with hematoxylin and eosin/periodic acid Schiff (H&E/PAS) and analyzed by light microscopy. A total histological score was calculated for each animal as described previously [24] and shown in Figure 2. Briefly, the samples were assigned a score (0–3 or 0–4) according to the severity (none, mild, moderate, severe) and extent (none, mucosal, submucosal, transmural) of inflammation, degree of crypt damage (basal 1/3 damaged, basal 2/3 damaged, crypts lost—epithelium intact, crypts lost—epithelium lost), and percentage of tissue affected (0, 1–25%, 26–50%, 51–75%, 76–100%).

The histological analyses were performed in a blinded fashion with respect to the treatment groups.

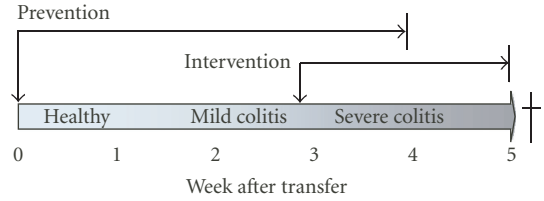


Figure 1: The adoptive transfer colitis model and treatment design. For prevention studies, the mice were treated from the day they were adoptively transferred with $CD4^{+}CD25^{-}$ T cells and until sacrificed when the disease is fully developed (three or four weeks after transfer). For the intervention studies, the treatment was initiated at week three after adoptive transfer. The treatment was continued for two weeks until sacrificed at week five.

*2.8. Statistical Analysis.* Fecal consistency score and histological score are shown as median (range) and analyzed using the Mann-Whitney $U$-test. WBC count, body weight at postmortem, and colonic weight : length ratio are shown as mean ± standard error of the mean (SEM) and analyzed by Student's $t$-test using Welch's correction for unequal variances. Differences were considered statistically significant when $P < 0.05$.

## 3. Results

In the following, the disease modifying effect of each of the investigated compounds in the adoptive transfer colitis model is presented. A schematic representation of the drug targets is shown in Figure 3. First, experimental data with the biologicals (i.e., monoclonal antibodies (mAb) and receptor fusion proteins (R-Fc)) are presented, followed by data from a number of compounds currently used to treat CD or UC. Although broad-spectrum antibiotics and metronidazole are mainly used for subgroups of IBD patients or for complications like pouchitis, we have included this treatment regimen, since the influence of the microflora

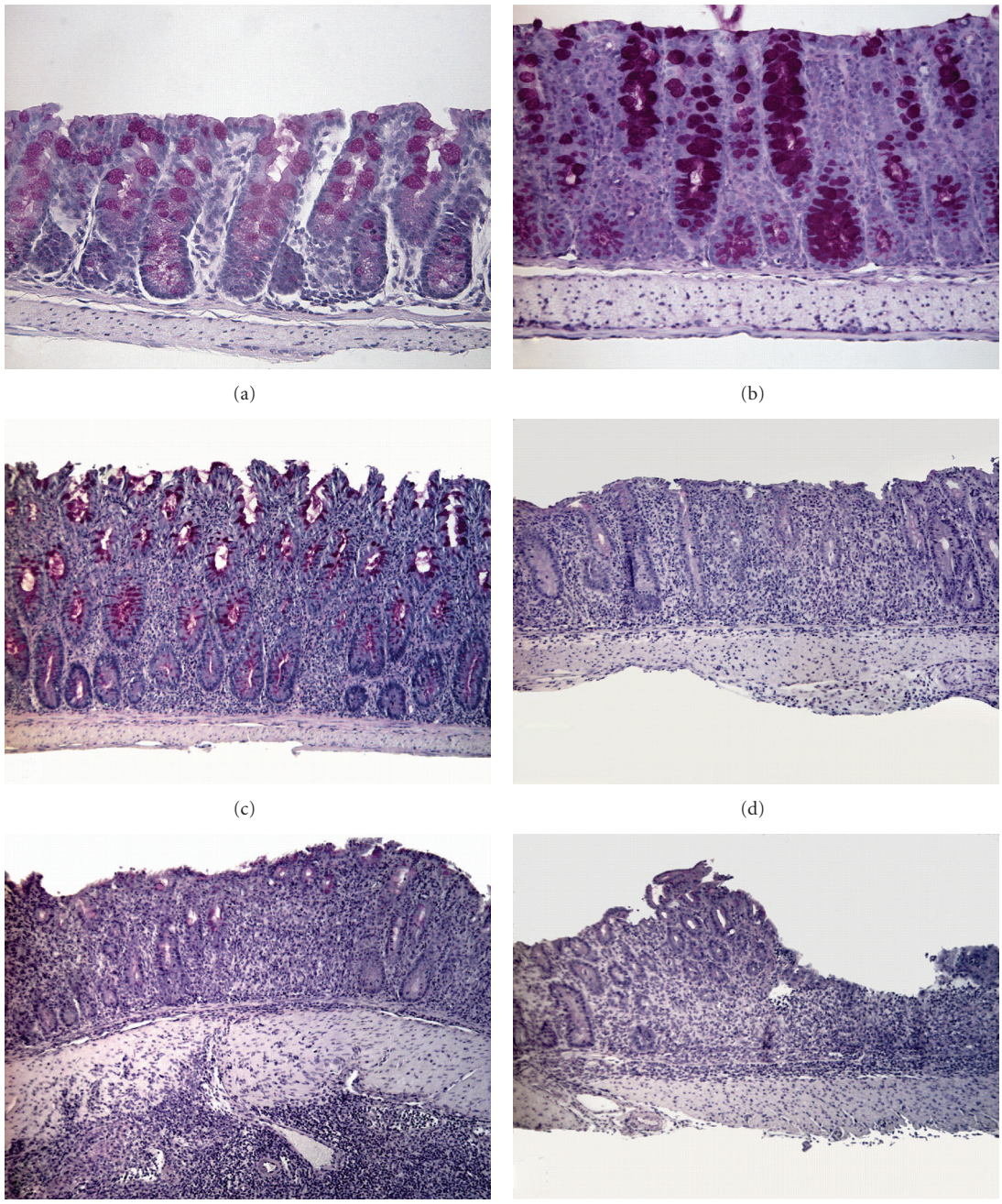


Figure 2: Histological changes in colon after adoptive transfer. Representative photomicrographs of histological changes leading to a progressively higher score from (a) to (f): (a) normal, (b) mild inflammation restricted to the mucosa, (c) moderate mucosal inflammation, (d) severe inflammation extending to submucosa, moderate to severe crypt degeneration, (e) severe transmural inflammation, moderate to severe crypt degeneration, (f) as (e) but with ulceration. Original magnification ×25 for (a-b) and ×10 for (c–f).

in the pathogenesis of IBD is a central topic. Due to the large data material, readers are referred to the supplementary material for a complete presentation of data (Figure SF1 and Tables ST1–ST10 available at doi: 10.1155/2012/412178).

*3.1. Rat Anti-Mouse TNF-α mAb Treatment.* In the 28 day prevention study, mice treated with the isotype control began to loose weight after two weeks, while the weight curve for the rat anti-mouse TNF-$\alpha$ mAb-treated mice was comparable to that of healthy controls. At the end of the study, the anti-TNF-$\alpha$-treated group had lost significantly less weight than the control group ($P < 0.001$, Figure 4, Tables 2 and ST1). Two mice in the control group were sacrificed due to extensive weight loss before the end of the study. The

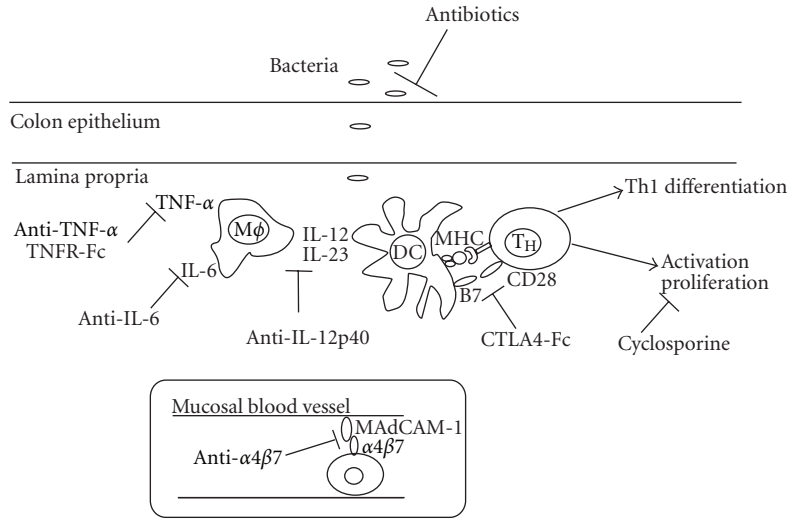


Figure 3: Inhibition of disease pathways in the adoptive transfer model. Schematic representation of drug targets.

Table 2: Statistics for all compounds. Clear lines represent prevention studies and bold represent intervention studies.

| Compound | Weight change | Fecal score | WBC count | Colonic weight:length | Histological score |
|---|---|---|---|---|---|
| Anti-TNF$\alpha$ | <0.001 | <0.001 | <0.001 | <0.001 | <0.0001 |
| | **<0.05** | **ns** | **ns** | **ns** | **ns** |
| TNFR-Fc[†] | <0.01 | ns | ns | ns | — |
| | **ns** | **ns** | **ns** | **ns** | **—** |
| Anti-IL-12p40 | <0.001 | ns | <0.05 | <0.001 | <0.001 |
| | **<0.0001** | **ns** | **0.001** | **<0.01** | **<0.05** |
| Anti-IL-6 | <0.01 | <0.01 | ns | <0.05 | <0.05 |
| | **ns** | **ns** | **ns** | **ns** | **ns** |
| CTLA4-Ig | <0.01 | <0.01 | <0.01 | <0.001 | <0.05* |
| | **<0.01** | **<0.01** | **<0.001** | **<0.001** | **<0.001** |
| Anti-$\alpha 4\beta 7$ | <0.0001 | <0.01 | ns | <0.01 | <0.01 |
| | **ns** | **ns** | **ns** | **ns** | **ns** |
| Enro + Metro | ns | <0.001 | <0.05 | <0.001 | <0.001 |
| | **<0.01** | **<0.001** | **<0.001** | **<0.0001** | **<0.001** |
| Cyclosporine | ns | ns | <0.05** | ns | ns |
| | — | — | — | — | — |

[†] Prevention = 50 mg/kg, intervention = 5 mg/kg.
* Used Wilcoxon signed rank test and compared with a hypothetical value of 0.0 since all scores were 0 in the CTLA4-Ig group.
** WBC count higher in treatment group—not analyzed.

fecal score was increased in both groups but was significantly lower in the anti-TNF-$\alpha$ group ($P < 0.001$, Tables 2 and ST2). The WBC count in the anti-TNF-$\alpha$ group was almost as low as in the unreconstituted controls, while it was significantly higher in the isotype control group ($P = 0.001$, Table 2 and ST3). Similarly, the colonic W : L ratio ($P < 0.001$) and histological score ($P < 0.0001$) were significantly lower in the anti-TNF-$\alpha$ group compared to the isotype controls (Tables 2, SF1, ST4-5). In contrast to the prevention studies, intervention therapy with anti-TNF-$\alpha$ did not consistently ameliorate colitis in this model. Although the anti-TNF-$\alpha$-treated group lost less weight than the control group ($P < 0.05$), none of the other clinical parameters were significantly affected by the treatment (Table 2 and ST6–10). Both for the fecal score, colonic W : L ratio and histological score (SF1), the group seemed equally divided into responders and non responders, that is, having high or low scores and values, respectively, rather than being equally distributed around the mean or median.

*3.2. TNF-α Receptor Fc Treatment.* We first tested the human TNFR-Fc fusion protein etanercept at a dose of 5 mg/kg in a 21 days prevention study and found no significant effect of the compound on any of the parameters analyzed (data

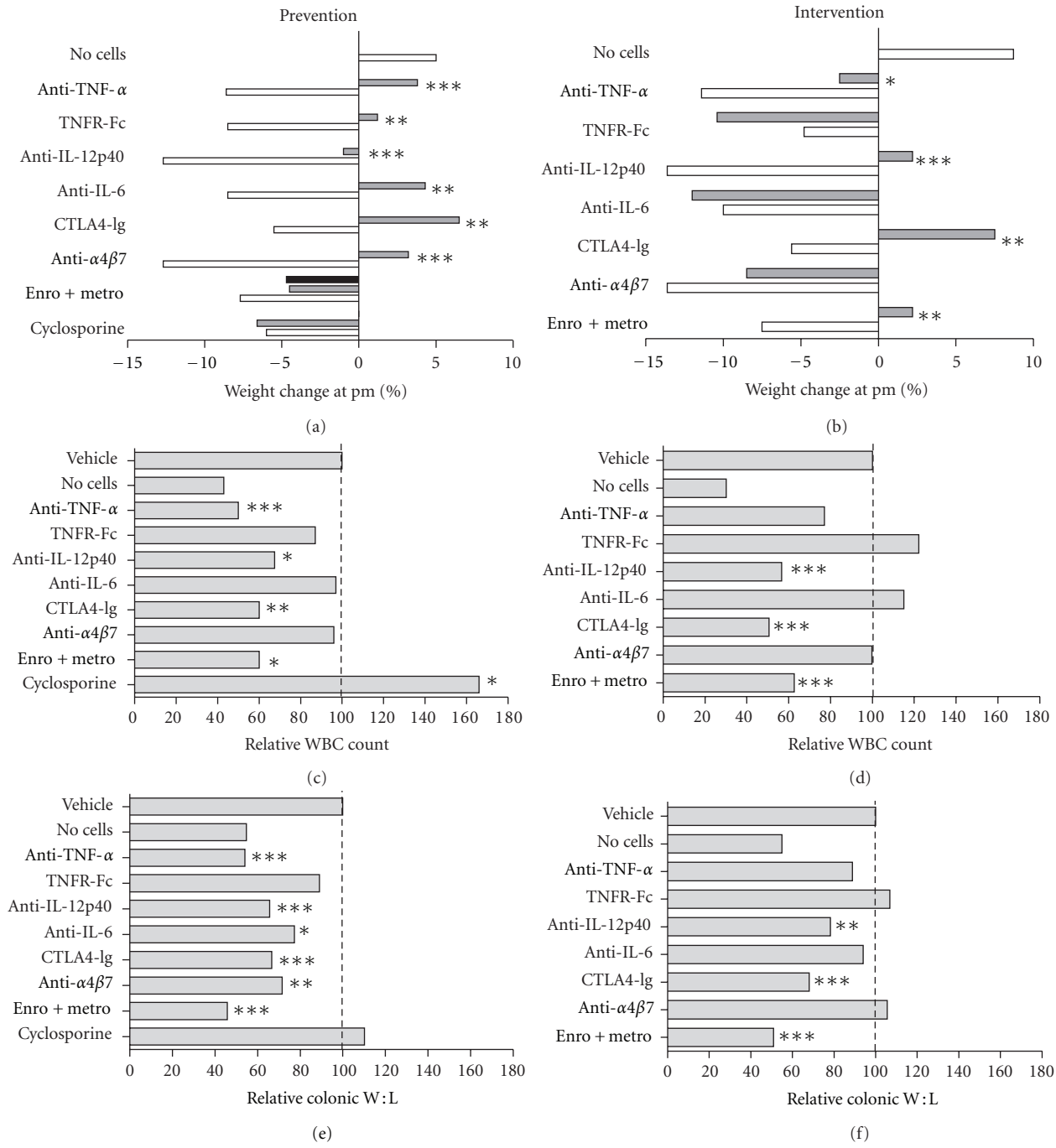


Figure 4: Changes in key disease parameters following treatment. Key disease parameters (weight loss, WBC counts, and colon W : L ratio) are depicted for preventive treatment (a–c) and interventive treatment (d–f). Disease parameters are shown as ((post mortem weight−start weight)/Start weight)∗100, (WBC count of drug/WBC count of control)∗100, (W : L ratio of drug/W : L ratio of control)∗100. (a and d) White bars represent vehicle control groups, grey bars represent treatment groups, and black bars represent mice which were not reconstituted but received treatment. (b, c, e, and f) Grey bars represent relative WBC counts and W : L ratios.

not shown). In the subsequent 28 days prevention study with a dose of 50 mg/kg, this group had significantly less weight loss than the vehicle control group ($P < 0.01$), while the fecal score was slightly but not significantly lower (Figure 4, Tables 2 and ST1-2). One mouse in the vehicle control group was sacrificed before week four, due to extensive weight loss. There was no difference in the mean WBC count or colonic W : L ratio between the two groups. Since we found no effects of the compound (except from decreased weight loss) and since the colonic W : L ratio is highly predictable for the histological score (as described in [15]), no histological scoring was made. In the intervention study,

TNFR-Fc (5 mg/kg) did not affect any of the measured parameters (Table 2 and ST6–10). Since the prevention study using 50 mg/kg did not have any effect either, this dose was not tested in intervention studies.

*3.3. Rat Anti-Mouse IL-12p40 mAb Treatment.* In the 28-day prevention study, mice treated with the isotype control began to loose weight after two weeks, while the weight curve for the rat anti-mouse IL-12p40 mAb-treated mice was comparable to that of healthy controls. At the termination of the study, mice treated with anti-IL-12p40 mAb had lost significantly less weight than the isotype control group ($P < 0.001$) (Table 2 and ST1). Similarly, anti-IL-12p40 mAb treatment resulted in significantly lower WBC count ($P < 0.05$), colonic W : L ratio and histological score (SF1) ($P < 0.001$ for both parameters), while it did not significantly improve fecal score (Tables 2 and ST2-S5). Intervention with anti-IL-12p40 mAb from day 21 reversed the progressive weight loss. At the end of the study, this group had lost significantly less weight than the control group ($P < 0.0001$) (Table 2 and ST6). The fecal score tended to be lower in the anti-IL-12p40 mAb group, and the colonic W : L ratio and histological score were significantly reduced compared to the isotype control ($P < 0.01$ and $P < 0.05$, resp., Tables 2, SF1, and ST7-10).

*3.4. Rat Anti-Mouse IL-6 mAb Treatment.* Preventive treatment with a rat anti-mouse IL-6 mAb reduced weight loss ($P < 0.01$) and fecal score ($P < 0.01$) but failed to significantly reduce WBC counts compared to the isotype control group (Table 2 and ST1–3). The colonic W : L ratio of the anti-IL-6 mAb treated group was significantly lower than that of the isotype treated group and was comparable to the unreconstituted healthy control group (Table 2 and ST4). Similarly, the histological score was significantly less in the anti-IL-6 mAb-treated group compared to the isotype control ($P < 0.01$, Tables 2, SF1, and ST5). Intervention with anti-IL-6 mAb from day 21 did not significantly affect any of the measured parameters (Tables 2, SF1, and S6-10).

*3.5. Human CTLA4-Ig Treatment.* Preventive treatment with human CTLA4-Ig effectively inhibited development of colitis. The weight curves for CTLA4-Ig-treated mice were comparable to the weight curves for the unreconstituted healthy control mice. The fecal score at the termination of the study ($P < 0.01$), the WBC count ($P < 0.01$), and colonic W : L ratio ($P < 0.001$) were significantly lower in the CTLA4-Ig groups compared to the isotype control group (Table 2 and ST1–4). Remarkably, no signs of inflammation were found in any of the animals, suggesting a very potent effect of the compound (Tables 2, SF1, and ST5).

A profound effect of CTLA4-Ig treatment on all the measured parameters was likewise identified in the intervention study. Shortly after initiation of treatment, the weight loss was reversed and the animals gained weight comparable to the unreconstituted. Likewise, the fecal score ($P < 0.01$), WBC count ($P < 0.001$), colonic W : L ratio ($P < 0.001$), and histological score ($P < 0.001$) were significantly lower than for the isotype control group at the end of the study (Tables 2, SF1, and ST6-10).

*3.6. Rat Anti-Mouse α4β7 Integrin mAb Treatment.* Preventive treatment with a rat anti-mouse $\alpha4\beta7$ mAb diminished weight loss ($P < 0.001$) and fecal score ($P < 0.05$, Table 2). However, the number of WBC was not significantly changed (Tables 2 and ST2-3). Inhibition of T-cell homing to the gut by anti-$\alpha4\beta7$ mAb treatment reduced colonic disease as indicated by the significantly lower colonic W : L ratio and histological score compared to the isotype control group ($P < 0.01$ for both parameters, Tables 2, SF1, and ST4-5). Intervention with anti-$\alpha4\beta7$ mAb at day 21 did not significantly affect any of the measured parameters (Figure 4, Tables 2, SF1, and ST6–10).

*3.7. Antibiotic (Enrofloxacin and Metronidazole) Treatment.* Mice treated with antibiotics (unreconstituted and reconstituted) did not develop weight loss in a preventive setting. In contrast, reconstituted vehicle treated mice progressively lost weight (Figure 4). The fecal score was slightly increased by the treatment itself but was significantly lower in the reconstituted mice treated with antibiotics compared to the reconstituted vehicle group ($P < 0.001$) as was the WBC count ($P < 0.05$), the colonic W : L ratio ($P < 0.001$), and the histological score ($P < 0.001$, Tables 2, SF1, and ST2-5).

Intervention with antibiotics immediately reversed weight loss (Figure 4). At the termination of the study, the mice treated with antibiotics had lost significantly less weight than the vehicle control group ($P < 0.01$). Likewise, fecal score ($P < 0.0001$), WBC count ($P < 0.001$), colonic W : L ratio ($P < 0.0001$), and histological score ($P < 0.001$) were significantly lower than for the vehicle control group (Figure 4, Tables 2, SF1, and S7–10).

*3.8. Cyclosporine Treatment.* Cyclosporine had no effect on the degree of weight loss, fecal score, colonic W : L ratio or histological score, while the WBC count at necropsy was actually significantly higher in the cyclosporine group compared to the vehicle group (Table 2, SF1, and ST1-5). Since there were no effects of cyclosporine in the prevention study, the compound was not tested in intervention studies.

*3.9. Summary of Experimental Data.* Collectively, we found that CTLA4-Ig, anti-IL-12p40 mAb, and antibiotics prevented onset of colitis and cured established disease, while anti-TNF-$\alpha$ mAb, anti-IL-6 mAb, and anti-$\alpha4\beta7$ mAb prevented onset of colitis but did not reverse established disease. Neither TNFR-Fc nor cyclosporine had any preventive or therapeutic effect in the current setup.

## 4. Discussion

The search for improved treatment opportunities against IBD is heavily dependent upon good animal models, both for efficacy studies and for understanding the underlying cause of the disease. To have any predictive value, the models must share central drivers of disease with the human disease they

are representing. Intervention with some of the known main mechanisms in autoimmune disease (i.e., costimulation, T-cell homing, effect of cytokines, etc.) can teach us more about which pathways are central for the model. This allows one to select a model appropriate for the inflammatory pathway, the test compound is supposed to act on. The aim of our study was to estimate the preventive and therapeutic effect of a number of established or potential IBD drugs, using the SCID adoptive transfer colitis model. By using multiple drugs inhibiting potential or known pathogenic drivers in IBD, we sought after an increased understanding of the central drivers, in this specific model. Several of these compounds or their surrogate antibodies have previously been tested in colitis models, but never in the same study with essentially similar experimental setup and with compounds which are all commercially available. This allows a more precise comparison of the different compounds and thereby a better assessment of the model's predictive value. We chose the adoptive transfer colitis model not only because of its similarities with IBD (mainly CD), but also because the model has several practical advantages compared to other chronic colitis models in relation to pharmacological testing (e.g., the synchronized onset of disease, no generation of anti-drug antibodies and commercial availability of mice).

TNF-$\alpha$ is a key proinflammatory cytokine in IBD. The cytokine exerts its effects via activation of NF$\kappa$B and MAPK pathways, and subsequently induction of IL-6 and IL-1b, inhibition of T-cell apoptosis, chemoattraction, and so forth [25]. We found a significant preventive treatment effect of anti-TNF-$\alpha$ mAb, as has been previously described in the $CD45RB^{High}$ model [11]. Our model setup suggests that TNF-$\alpha$ is most important in the beginning of disease since anti-TNF-$\alpha$ was largely effective in the prevention study. It is possible that the redundancy of the inflammatory cascades makes TNF-$\alpha$ less important when the inflammation is already established and $CD4^+$ T cells have differentiated into a pathogenic effector phenotype. However, the human anti-TNF-$\alpha$ mAb's (adalimumab and infliximab) can induce remission in the majority of CD patients. These mAbs' have been reported to neutralize TNF-$\alpha$ as well as to induce apoptosis in T cells [26]. Whether the surrogate rat anti-mouse TNF-$\alpha$ mAb also has this dual function is unknown. As opposed to anti-TNF-$\alpha$ mAb treatment, there was no effect of TNFR-Fc in our studies, which is in accordance to the findings in CD [27].

IL-12p40 is predominantly produced by dendritic cells and phagocytes in response to microbial stimulation. It has a critical role in promoting the differentiation of naïve $CD4^+$ T cells into mature T-helper effector cells. It is currently believed that IL-12 (p23/p40) and IL-23 (p19/p40) are central for the Th1 and the Th17 pathways, respectively, and both cytokines are inhibited by the anti-IL-12p40 mAb [16, 28]. We found that the anti-IL-12p40 mAb treatment was effective in our prevention as well as intervention setup, suggesting the importance of these pathways in the transfer model. Our observation is in agreement with previous results [20, 29, 30]. Significant clinical responses following treatment with an anti-IL-12p40 mAb have also been reported in CD patients [5, 31], and the drug is currently recruiting for a phase III trial in CD. Thus, our results suggest that the SCID adoptive transfer model is suitable for studying compounds targeting the IL-12p40 pathways.

IL-6 is a pleiotropic cytokine with a central role in immune regulation. Increased serum concentrations of IL-6 were reported to correlate with clinical activity of CD, and antibodies targeting IL-6 or IL-6R were efficacious in several animal models [16, 32, 33]. In accord, a humanized mAb against IL-6R showed promising results in a phase II study in active CD [6]. We found a significant therapeutic effect of anti-IL-6 in a preventive setting but not in the intervention study. As for the anti-TNF-$\alpha$ mAb treatment, this suggests that IL-6 is most important in the beginning, while the redundancy of the inflammatory cascades may make IL-6 less important when the inflammation is already established.

CTLA4-Ig binds to CD80/86 thereby preventing costimulation of T cells via CD28 [34]. CTLA4-Ig has shown therapeutic efficacy in several autoimmune diseases including rheumatoid arthritis, and in experimental models of autoimmune diseases [34, 35]. We found that CTLA4-Ig completely prevented colitis and very efficiently cured established disease, indicating a central role of T-cell costimulation in the model. However, shortly after completion of our experimental studies, clinical trials with CTLA4-Ig in UC and CD were terminated due to lack of efficacy [36]. The lack of negative regulation through CTLA-4 on, for example, Tregs may yield a therapeutic window which is not present in humans. Thus, the lack of important self-regulatory mechanisms must be taken into account when evaluating drugs targeting this specific pathway.

Neutralization of the integrin $\alpha4\beta7$ on lymphocytes and monocytes inhibits homing of the cells to the gut via mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on endothelial cells. We found that this could prevent the onset of disease in the transfer model as demonstrated previously [37]. However, once the colitis was already established, recruitment of leucocytes to the colon via this mechanism seemed no longer essential for maintenance of disease in our experimental setup. In contrast, resolution of established disease has been demonstrated in the cotton-top tamarin (spontaneous colitis) [38].

In phase II studies, Vedolizumab (targeting human $\alpha4\beta7$) failed to meet the primary endpoint in CD patients with active disease, while there was a significant therapeutic effect in patients with UC [39, 40]. Natalizumab, which neutralizes $\alpha4$ in conjunction with $\beta7$ as well as with $\beta1$, was effective for CD and is approved by the FDA to use in patients refractory to treatment with anti-TNF-$\alpha$ [41].

Thus, in IBD as well as in the models, there is not a clear-cut effect of inhibiting leukocyte homing via anti-$\alpha4\beta7$, although it seems more effective in man than in the SCID transfer model. This could be due to the slower progression of the human disease, that is, there is a larger "window open for treatment" where recruitment of cells to the gut is still important. An alternative explanation is the involvement of $\alpha4\beta7$ in homing to the small bowel and Payer's patches in Crohn's disease compared to $\alpha4\beta7$ involvement in colonic $CD4^+$ T cell infiltration in the SCID adoptive transfer model.

Although anti-$\alpha 4\beta 7$ could not reverse established colitis in the model, the marked effect of the antibody in prevention studies suggests that the model is useful in studies of leukocyte homing to the colon via integrins and adhesion molecules.

Cyclosporine inhibits calcineurin, thereby inhibiting T-cell activation and proliferation. The compound is effective in patients with severe steroid-refractory UC [7, 42], while no controlled studies have shown effect of cyclosporine in CD [43]. We did not find any effect of preventive treatment with cyclosporine in accord with previous studies in transfer models [35, 44]. In contrast, cyclosporine significantly ameliorated acute DSS-induced colitis [19, 45]. Considering that the DSS model is mainly UC-like and the transfer model mainly CD-like (at least in terms of Th1/TH17 immunopathogenesis), data from experimental models are translational to the human disease. It is not clear why cyclosporine lacks effect in CD and in our model, but it has previously been shown that T cells are able to proliferate and exert effector functions via cyclosporine-resistant mechanisms [46–48]. In addition to its effect on T cells, cyclosporine has been shown to inhibit the effect of several other immune cells and proinflammatory mechanisms, which may account for its therapeutic effects in the acute DSS model, where T cells are not required for development of disease [49].

The normal intestinal microbial flora (microbiota) contributes significantly to the etiopathogenesis of IBD [3, 50], and antibiotics have in some studies been shown to induce and maintain remission in IBD patients [50]. However, the side-effects and the risk of developing microbial resistance associated with long-term treatment with antibiotics prevent the use of this treatment strategy. Instead, there is focus on developing microbial cultures, which can help bringing back the balance between the microbiota and the immune system [50]. We found that the combination of enrofloxacin and metronidazole completely prevented onset of colitis and cured established colitis in the adoptive transfer model, emphasizing the significance of the microbiota in this model. Thus, the experimental model and the human disease share this central factor in the immunopathogenesis, suggesting that the model may be useful in studies of the microflora's impact on disease. Combined treatment with metronidazole, which kills anaerobes and influences cell trafficking [51], was essential since enrofloxacin alone did not affect the disease (data not shown).

## 5. Concluding Remarks

We have evaluated the therapeutic effect of a number of IBD drugs and drug candidates in the SCID adoptive transfer colitis model and compared this to the therapeutic effect in IBD patients, when this information was available. The study shows that certain drivers of inflammation are shared between the model and the human diseases, that is, the cytokines IL-12p40, TNF-$\alpha$ and IL-6, the homing molecule $\alpha 4\beta 7$, and the microbial flora. With regards to these drivers, the model seems to have a good predictive value.

However, our studies indeed also show limitations of the adoptive transfer model in this respect, since not all drugs effective against IBD are effective in the model. Following adoptive transfer, the development of disease is driven by the extreme expansion of the $CD4^+$ cells in a lymphopenic host, and neither B cells nor $CD8^+$ cells are present. Thus, some treatment effects in the preventive studies might be due to interfering with homeostatic expansion, which is not relevant in IBD and conversely, a test compound's effect on B and $CD8^+$ T cells will have no effect in the model, although it might be effective in IBD. Moreover, compared to clinical trials where efficacy is evaluated, for example, 4–18 weeks post-initiation of treatment for some test compounds, an intervention period of two weeks may be too short to obtain a therapeutic effect in the model. This could be addressed with a model where the disease is progressing more slowly. This stresses the need of critically choosing for which types of experiments and compounds to use animal models and which of the models to use. It should be noted that there is a great variability in the disease pattern in IBD patients, and that patients respond differently to medication. The various experimental colitis models may represent different types and stages of severity of UC or CD [13] and altogether this makes the translation of data from experimental models to humans even more challenging.

## Abbreviations

| | |
|---|---|
| CD: | Crohn's disease |
| CTLA-4: | Cytotoxic T-Lymphocyte Antigen 4 |
| GM-CSF: | Granulocyte macrophage colony-stimulating factor |
| Ig: | Immunoglobulin |
| IBD: | Inflammatory bowel disease |
| IL: | Interleukin |
| IFN-$\gamma$: | Interferon-gamma |
| cIg: | Isotype controls |
| ns: | Not significant |
| TNF-R: | Tumor necrosis factor receptor |
| TNF: | Tumor necrosis factor |
| UC: | Ulcerative colitis |
| W : L: | Weight-to-length ratio |
| WBC: | White blood cell. |

## References

[1] J. Loftus, "Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences," *Gastroenterology*, vol. 126, no. 6, pp. 1504–1517, 2004.

[2] F. Casellas, J. López-Vivancos, X. Badia, J. Vilaseca, and J. R. Malagelada, "Influence of inflammatory bowel disease on different dimensions of quality of life," *European Journal of Gastroenterology and Hepatology*, vol. 13, no. 5, pp. 567–572, 2001.

[3] G. Bouma and W. Strober, "The immunological and genetic basis of inflammatory bowel disease," *Nature Reviews Immunology*, vol. 3, no. 7, pp. 521–533, 2003.

[4] G. W. Dryden, "Overview of biologic therapy for Crohn's disease," *Expert Opinion on Biological Therapy*, vol. 9, no. 8, pp. 967–974, 2009.

[5] W. J. Sandborn, B. G. Feagan, R. N. Fedorak et al., "A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease," *Gastroenterology*, vol. 135, no. 4, pp. 1130–1141, 2008.

[6] J. Bilsborough and J. L. Viney, "From model to mechanism: lessons of mice and men in the discovery of protein biologicals for the treatment of inflammatory bowel disease," *Expert Opinion on Drug Discovery*, vol. 1, no. 1, pp. 69–83, 2006.

[7] D. C. Baumgart and W. J. Sandborn, "Inflammatory bowel disease: clinical aspects and established and evolving therapies," *Lancet*, vol. 369, no. 9573, pp. 1641–1657, 2007.

[8] W. Strober, I. J. Fuss, and R. S. Blumberg, "The immunology of mucosal models of inflammation," *Annual Review of Immunology*, vol. 20, pp. 495–549, 2002.

[9] P. J. Morrissey, K. Charrier, S. Braddy, D. Liggitt, and J. D. Watson, "$CD4^+$ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified $CD4^+$ T cells," *Journal of Experimental Medicine*, vol. 178, no. 1, pp. 237–244, 1993.

[10] F. Powrie, M. W. Leach, S. Mauze, L. B. Caddle, and R. L. Coffman, "Phenotypically distinct subsets of $CD4^+$ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice," *International Immunology*, vol. 5, no. 11, pp. 1461–1471, 1993.

[11] F. Powrie, M. W. Leach, S. Mauze, S. Menon, L. B. Caddle, and R. L. Coffman, "Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with $CD45RB^{hi}$ $CD4^+$ T cells," *Immunity*, vol. 1, no. 7, pp. 553–562, 1994.

[12] S. Hue, K. J. Maloy, B. McKensie, D. Cua, and F. Powrie, "IL-23 and not IL-12 is essential for the development of IBD," *Inflammatory Bowel Diseases*, vol. 12, supplement 2, S25 pages, 2006.

[13] H. H. Uhlig and F. Powrie, "Mouse models of intestinal inflammation as tools to understand the pathogenesis of inflammatory bowel disease," *European Journal of Immunology*, vol. 39, no. 8, pp. 2021–2026, 2009.

[14] J. L. Coombes, N. J. Robinson, K. J. Maloy, H. H. Uhlig, and F. Powrie, "Regulatory T cells and intestinal homeostasis," *Immunological Reviews*, vol. 204, pp. 184–194, 2005.

[15] S. Kjellev, D. Lundsgaard, S. S. Poulsen, and H. Markholst, "Reconstitution of Scid mice with $CD4^+CD25^-$ T cells leads to rapid colitis: an improved model for pharmacologic testing," *International Immunopharmacology*, vol. 6, no. 8, pp. 1341–1354, 2006.

[16] D. Yen, J. Cheung, H. Scheerens et al., "IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6," *Journal of Clinical Investigation*, vol. 116, no. 5, pp. 1310–1316, 2006.

[17] D. Teoh, L. A. Johnson, T. Hanke, A. J. McMichael, and D. G. Jackson, "Blocking development of a $CD8^+$ T cell response by targeting lymphatic recruitment of APC," *Journal of Immunology*, vol. 182, no. 4, pp. 2425–2431, 2009.

[18] B. R. Lúdvíksson, W. Strober, R. Nishikomori, S. K. Hasan, and R. O. Ehrhardt, "Administration of mAb against $\alpha(E)\beta_7$ prevents and ameliorates immunization-induced colitis in IL-2(-/-) mice," *Journal of Immunology*, vol. 162, no. 8, pp. 4975–4982, 1999.

[19] S. Melgar, L. Karlsson, E. Rehnstrøm et al., "Validation of murine dextran sulfate sodium-induced colitis using four therapeutic agents for human inflammatory bowel disease," *International Immunopharmacology*, vol. 8, no. 6, pp. 836–844, 2008.

[20] N. J. Davidson, S. A. Hudak, R. E. Lesley, S. Menon, M. W. Leach, and D. M. Rennick, "IL-12, but not IFN-$\gamma$, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice," *Journal of Immunology*, vol. 161, no. 6, pp. 3143–3149, 1998.

[21] J. Kim, C. K. Chang, T. Hayden et al., "The activating immunoreceptor NKG2D and its ligands are involved in allograft transplant rejection," *Journal of Immunology*, vol. 179, no. 10, pp. 6416–6420, 2007.

[22] H. L. Plessner, P. L. Lin, T. Konno et al., "Neutralization of Tumor Necrosis Factor (TNF) by antibody but not TNF receptor fusion molecule exacerbates chronic murine tuberculosis," *Journal of Infectious Diseases*, vol. 195, no. 11, pp. 1643–1650, 2007.

[23] S. S. Kang, S. M. Bloom, L. A. Norian et al., "An antibiotic-responsive mouse model of fulminant ulcerative colitis," *PLoS Medicine*, vol. 5, no. 3, article e41, 2008.

[24] K. L. Williams, C. R. Fuller, L. A. Dieleman et al., "Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone," *Gastroenterology*, vol. 120, no. 4, pp. 925–937, 2001.

[25] F. Sanchez-Muñoz, A. Dominguez-Lopez, and J. K. Yamamoto-Furusho, "Role of cytokines in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 14, no. 27, pp. 4280–4288, 2008.

[26] R. Atreya, M. Zimmer, B. Bartsch et al., "Anti-TNF antibodies target T-cell apaotosis in inflammatory bpwel diseases via TNFR2 and intestinal $CD14^+$ macrophages," *Gastroenterology*, vol. 141, no. 6, pp. 1026–1038, 2011.

[27] W. J. Sandborn, S. B. Hanauer, S. Katz et al., "Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial," *Gastroenterology*, vol. 121, no. 5, pp. 1088–1094, 2001.

[28] G. Trinchieri, "Interleukin-12 and the regulation of innate resistance and adaptive immunity," *Nature Reviews Immunology*, vol. 3, no. 2, pp. 133–146, 2003.

[29] Z. Liu, K. Geboes, H. Heremans et al., "Role of interleukin-12 in the induction of mucosal inflammation and abrogation of regulatory T cell function in chronic experimental colitis," *European Journal of Immunology*, vol. 31, no. 5, pp. 1550–1560, 2001.

[30] M. F. Neurath, "IL-23: a master regulator in Crohn disease," *Nature Medicine*, vol. 13, no. 1, pp. 26–28, 2007.

[31] P. J. Mannon, I. J. Fuss, L. Mayer et al., "Anti-interleukin-12 antibody for active Crohn's disease," *New England Journal of Medicine*, vol. 351, no. 20, pp. 2069–2079, 2004.

[32] M. Yamamoto, K. Yoshizaki, T. Kishimoto, and H. Ito, "IL-6 is required for the development of Th1 cell-mediated murine colitis," *Journal of Immunology*, vol. 164, no. 9, pp. 4878–4882, 2000.

[33] R. Atreya, J. Mudter, S. Finotto et al., "Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo," *Nature Medicine*, vol. 6, no. 5, pp. 583–588, 2000.

[34] P. S. Linsley and S. G. Nadler, "The clinical utility of inhibiting $CD28^-$mediated costimulation," *Immunological Reviews*, vol. 229, no. 1, pp. 307–321, 2009.

[35] C. M. Davenport, H. A. McAdams, J. Kou et al., "Inhibition of pro-inflammatory cytokine generation by CTLA4-Ig in the

skin and colon of mice adoptively transplanted with CD45RB$^{hi}$ CD4$^{+}$ T cells correlates with suppression of psoriasis and colitis," *International Immunopharmacology*, vol. 2, no. 5, pp. 653–672, 2002.

[36] http://www.bms.com/clinical_trials/results/Pages/default.aspx, 2011.

[37] D. Picarella, P. Hurlbut, J. Rottman, X. Shi, E. Butcher, and D. J. Ringler, "Monoclonal antibodies specific for $\beta$ 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RB$^{high}$ CD4$^{+}$ T cells," *Journal of Immunology*, vol. 158, no. 5, pp. 2099–2106, 1997.

[38] S. Wirtz and M. F. Neurath, "Animal models of intestinal inflammation: new insights into the molecular pathogenesis and immunotherapy of inflammatory bowel disease," *International Journal of Colorectal Disease*, vol. 15, no. 3, pp. 144–160, 2000.

[39] B. G. Feagan, G. R. Greenberg, G. Wild et al., "Treatment of ulcerative colitis with a humanized antibody to the $\alpha 4\beta 7$ integrin," *New England Journal of Medicine*, vol. 352, no. 24, pp. 2499–2507, 2005.

[40] B. G. Feagan, G. R. Greenberg, G. Wild et al., "Treatment of active Crohn's disease with MLN0002, a humanized antibody to the $\alpha 4\beta 7$ integrin," *Clinical Gastroenterology and Hepatology*, vol. 6, no. 12, pp. 1370–1377, 2008.

[41] D. K. Podolsky, "Beyond tumor necrosis factor: next-generation biologic therapy for inflammatory bowel disease," *Digestive Diseases*, vol. 27, no. 3, pp. 366–369, 2009.

[42] R. R. Cima and J. H. Pemberton, "Medical and surgical management of chronic ulcerative colitis," *Archives of Surgery*, vol. 140, no. 3, pp. 300–310, 2005.

[43] J. W. McDonald, B. G. Feagan, D. Jewell, J. Brynskov, E. F. Stange, and J. K. Macdonald, "Cyclosporine for induction of remission in Crohn's disease," *Cochrane Database of Systematic Reviews*, no. 2, pp. CD000297–CD002005, 2005.

[44] Y. Ikenoue, T. Tagami, and M. Murata, "Development and validation of a novel IL-10 deficient cell transfer model for colitis," *International Immunopharmacology*, vol. 5, no. 6, pp. 993–1006, 2005.

[45] S. N. Murthy, H. S. Cooper, H. Shim, R. S. Shah, S. A. Ibrahim, and D. J. Sedergran, "Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin," *Digestive Diseases and Sciences*, vol. 38, no. 9, pp. 1722–1734, 1993.

[46] G. M. Pereira, J. F. Miller, and E. M. Shevach, "Mechanism of action of cyclosporine A in vivo. II. T cell priming in vivo to alloantigen can be mediated by an IL-2-independent cyclosporine A-resistant pathway," *Journal of Immunology*, vol. 144, no. 6, pp. 2109–2116, 1990.

[47] I. Motta, J. H. Colle, B. Shidani, and P. Truffa-Bachi, "Interleukin 2/interleukin 4-independent T helper cell generation during an in vitro antigenic stimulation of mouse spleen cells in the presence of cyclosporin A," *European Journal of Immunology*, vol. 21, no. 3, pp. 551–557, 1991.

[48] J. D. Fayen, "Multiple cytokines sharing the common receptor $\gamma$ chain can induce CD154/CD40 ligand expression by human CD4$^{+}$ T lymphocytes via a cyclosporin A-resistant pathway," *Immunology*, vol. 104, no. 3, pp. 299–306, 2001.

[49] J. Kountouras, C. Zavos, and D. Chatzopoulos, "Immunomodulatory benefits of cyclosporine A in inflammatory bowel disease," *Journal of Cellular and Molecular Medicine*, vol. 8, no. 3, pp. 317–328, 2004.

[50] K. Mitsuyama and M. Sata, "Gut microflora: a new target for therapeutic approaches in inflammatory bowel disease," *Expert Opinion on Therapeutic Targets*, vol. 12, no. 3, pp. 301–312, 2008.

[51] H. Arndt, K. D. Palitzsch, M. B. Grisham, and D. N. Granger, "Metronidazole inhibits leukocyte-endothelial cell adhesion in rat mesenteric venules," *Gastroenterology*, vol. 106, no. 5, pp. 1271–1276, 1994.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 574079, 5 pages
doi:10.1155/2012/574079

*Research Article*

# Inflammatory Bowel Disease in Hispanics: The University of Puerto Rico IBD Registry

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Received 12 September 2011; Revised 29 October 2011; Accepted 7 November 2011

Academic Editor: David Sachar



A registry of patients with inflammatory bowel diseases, ulcerative colitis (UC) and Crohn's disease (CD), was created at the University of Puerto Rico in 1995. Subjects with a documented diagnosis of IBD by clinical, radiologic, endoscopic, and/or pathologic criteria were recruited from the IBD clinics, support groups, and community practices, and demographic and medical data was collected. All entries from 1995 to 2009 were analyzed for demographics, family history, disease extent, extraintestinal manifestations, surgery, and smoking history. Results were described using summary statistics. 635 Hispanics living in Puerto Rico, 299 with UC and 336 with CD, were included. Mean ages were 40.3 for UC and 30.9 for CD. Over half (56%) of UC and 41% of CD were females. Family history was present in 19.3% of UC and 17.5% of CD. Surgery for IBD had been performed in 31.9% of UC and 51.2% of the CD patients. Over one-fourth of the patients reported extraintestinal manifestations, most frequently arthropathies. Our findings contribute to the limited epidemiologic and clinical data on Hispanics with IBD.

## 1. Introduction

The study of Crohn's (CD) and ulcerative colitis (UC) has been focused on Caucasians, as the incidence and diagnosis of inflammatory bowel disease (IBD) have been more evident in this population. The rates of UC and CD are highest in northern climates, in urban regions, and in well-developed areas of the world such as North America and Europe and lowest in southern climates and underdeveloped areas [1–4]. The reported prevalence of IBD in adults in the United States (US) has ranged from 37 to 238 per 100,000 for UC and 26 to 201 per 100,000 for CD [4, 5].

The epidemiology of IBD seems to be changing. Incidence in North America, Northern and Western Europe has stabilized, and low incidence areas are showing an increase [4]. The racial and ethnic differences also seem to be narrowing. Reports of IBD from Barbados and the French West Indies in the Caribbean illustrate these changes [6, 7].

There is an increasing recognition of IBD in the minority populations of the United States [7–16], and several studies have described the epidemiology of IBD in these populations. Many of these studies have centered on blacks with IBD, while IBD in Hispanics is less well defined. The incidence and prevalence of IBD in Puerto Rico has been rising over the past decades [17–19]. With the aim of further examining Hispanics with IBD, we describe the demographic and clinical characteristics of a large university-based registry of IBD in Hispanics living in Puerto Rico.

## 2. Materials and Methods

The University of Puerto Rico IBD Registry was created in 1995 [17]. The Registry is a database of patients with UC and CD that collects demographic and medical information at the time of interview. Subjects with documented diagnosis of IBD by clinical, radiologic, endoscopic, and/or pathologic criteria are recruited from the IBD clinics, support group, and community practices and represent diverse areas of Puerto Rico. After informed consent, data is collected

through an interview by trained investigators and medical records review. Information collected includes age, gender, diagnosis, age at onset of symptoms, age at diagnosis, urban or rural living, place of birth and parentage, family history of IBD, smoking history, extraintestinal manifestations, extent of disease, medications, and surgery. Method of diagnosis, including endoscopic procedures, imaging, pathologic specimens, and surgical findings, is obtained from the treating physician's and/or clinic medical record. The diagnosis has to be established or confirmed by a gastroenterologist. No followup is provided for updating data in the study. The database is entered into an Excel spreadsheet. The Registry has continuing Institutional Review Board approval from the University of Puerto Rico Medical Sciences Campus (protocol no. 1250195).

This report includes only Hispanics living in Puerto Rico. Hispanic subjects were identified by having both parents of Puerto Rican, Dominican, Venezuelan, or Cuban origin. Place of birth and childhood home are recorded in the Registry. Five subjects were excluded because of non-Hispanic origins (two from the United States, one from Nevis, one from Israel, and one with a German mother). The vast majority of subjects (585) were born in Puerto Rico of Puerto Rican parents. Forty-four were born in the United States, three in the Dominican Republic, three in Cuba, and one in Venezuela.

All entries from 1995 until 2009 were analyzed for demographics, family history, smoking history, disease extent, extraintestinal manifestations, and surgery. Disease duration at the time of inclusion in the Registry was calculated from the time of symptom onset and from the time of diagnosis. Medications used have been reported in a separate study [20]. Summary measures were used to describe the study group.

## 3. Results

Six hundred thirty-five Hispanic patients, 299 (47.1%) with UC and 336 (52.9%) with CD, were included in the Registry during the study period. The ages ranged from 10 to 85 years old, with the youngest recording age of diagnosis at 7 years old. Table 1 shows the demographics, family history, and smoking history of the population included in this study. Patients with UC were older, predominantly female, and smoked less at the time of diagnosis, when compared to patients with CD. Mean age for UC patients was 40.3 ± 15.6 with mean ages at onset of 31 ± 14.4 and at diagnosis of 32.6 ± 14.4. Mean age for CD patients was 30.9 ± 12.2, with a mean age at onset of 24.7 ± 11.7 and at diagnosis of 26.8 ± 12.6. For ulcerative colitis, interval from onset was 9.3 years and interval from diagnosis was 7.7 years. For Crohn's disease, interval from onset was 6.2 years and interval from diagnosis was 4.1 years. More than half (56%) of UC patients were females, whereas 59% of CD patients were males. Nearly 12% of IBD patients were smokers at the time of diagnosis.

Family history of IBD was present in 19.3% of UC and 17.5% of CD patients. In the cohort with UC, there were 28 firstdegree relatives with UC and 4 with CD. An additional 27 other relatives had UC and 4 had CD. For patients with CD, 17 first-degree relatives (parents, siblings, or offspring) were affected with CD and 28 with UC. Twenty-six more remote relatives (including grandparents, aunts/uncles, cousins and nephews/nieces) had CD and 27 had UC.

Table 1: General characteristics of the Registry patients.

| | UC | CD |
|---|---|---|
| *n* | 299 | 336 |
| Male : female (%) | 44 : 56 | 59 : 41 |
| Mean age | 40.3 ± 15.6 | 30.9 ± 12.2 |
| Mean age onset | 31 ± 14.4 | 24.7 ± 11.7 |
| Mean age dx | 32.6 ± 14.4 | 26.8 ± 12.6 |
| Family history IBD *n* (%) | 58 (19.3%) | 59 (17.5%) |
| Smoking at dx (%) | 10% | 13.7% |

CD: Crohn's disease; UC: ulcerative colitis; IBD: inflammatory bowel disease.

Table 2: Extraintestinal manifestations.

| | UC (%) | CD (%) | Total IBD (%) |
|---|---|---|---|
| EIM | 78 (26) | 93 (27.6) | 171 (26.9) |
| All arthropathies | 58 (19.4) | 76 (22.8) | 134 (21.1) |
| Peripheral arthropathy | 48 (16.1) | 62 (18.4) | 110 (17.3) |
| Ankylosing spondylitis | 2 (0.7) | 1 (0.3) | 3 (0.5) |
| Sacroiliitis | 8 (2.6) | 13 (3.8) | 21 (3.3) |
| Erythema nodosum* | 5 (1.6) | 23 (6.8) | 28 (4.4) |
| Pyoderma gangrenosum | 6 (2) | 3 (0.9) | 9 (1.4) |
| Uveitis and/or episcleritis | 5 (1.7) | 10 (3) | 15 (2.4) |
| Primary sclerosing cholangitis | 3 (1.0) | 2 (0.6) | 5 (0.8) |
| Osteoporosis | 6 (2.0) | 14 (4.1) | 20 (3.1) |

* $P < 0.002$.

At the time of inclusion in the UPR IBD Registry, the disease extent in subjects with UC was as follows: 13% proctitis, 16.2% proctosigmoiditis, 21% left side colitis, and 49.6% pancolitis. In subjects with CD, 54.4% had ileal disease, 47% had colon involvement, 15.6% had perianal disease, 6.7% had jejunal disease, and 1.1% had upper gastrointestinal tract involvement. 31.9% of UC and over half (51.2%) of CD patients reported having surgery for IBD at the time of the interview.

Extraintestinal manifestations, reported in 171 patients, are shown in Table 2. The most common were the various arthropathies, similar in both UC and CD, followed by erythema nodosum, which was more frequent in CD (1.6% in UC versus 6.8% in CD, $P < 0.002$). Osteoporosis was found more frequently in CD, though not statistically significant (14 of 20 cases). Ophthalmologic manifestations were grouped together, as they were infrequent.

## 4. Discussion

Incidence and prevalence studies for Puerto Rico have shown an increase in both ulcerative colitis and Crohn's disease since the late 1990s [17–19].

Table 3: Crohn's disease: comparison with other Hispanic populations.

| | UPR Registry | PR (NIDDK-IBDGR) [7] | Spain [21] | Portugal [22] | Huelva, Spain [23] |
|---|---|---|---|---|---|
| *n* | 336 | 106 | 635 | 1692 | 30 |
| Male : female (%) | 59 : 41 | | 48 : 52 | 44 : 56 | 57 : 43 |
| Mean age | 30.9 | | 33 | 31 | |
| EIM (%) | 27.6 | | | | 36.7 |
| Fam hx IBD (%) | 17.5 | 16.6 | 15 | 5.8 | 33.3 |

Table 4: UC: comparison with other Hispanic populations.

| | UPR Registry | PR (NIDDK IBDGRC) [7] | Mexico [24] | Panama [25] | Argentina [25] F | Huelva, Spain [23] |
|---|---|---|---|---|---|---|
| *n* | 299 | 62 | 848 | 15 | 38 | 40 |
| Male : female (%) | 44 : 56 | | 45 : 55 | 60 : 40 | 39 : 61 | 45 : 55 |
| Mean age | 40.3 | | 31.3 | 38 | 45 | |
| EIM (%) | 26 | | 41.5 | | | 12.5 |
| Fam hx IBD (%) | 19.3 | 14.5 | 6.7 | | | 12.5 |

The clinical presentation of IBD in Hispanics has not been well studied. Most of the published studies focus on incidence and prevalence with limited descriptions of clinical characteristics. Studies in which Hispanics with IBD are compared with Caucasians and other ethnic or racial populations are limited by the small number of Hispanics included. A study of 148 patients with IBD seen from 1999 to 2003 compared 58 Whites, 54 African Americans, and 30 Mexican Americans. Family history was present in only 10% of Mexican Americans as compared to 33% of Whites. In those with ulcerative colitis, no differences in treatment and surgery were found [13]. In another study comparing the same three groups (H, AA, W), there was no difference between African Americans and Mexican Americans when separately compared to Whites in terms of intestinal manifestations of CD and UC, respectively. Among UC patients, Whites had significantly higher incidence than Mexican Americans of joint symptoms ($P < 0.0001$) and osteoporosis ($P = 0.001$). Whites had a stronger family history of IBD and colorectal carcinoma. All the Mexican Americans with UC who were tested had positive p-ANCA compared to only 40% of Whites ($P = 0.033$). Proctitis alone occurred in 32% of Whites, but only in 9% of Mexican Americans with UC ($P = 0.022$). In general, there were no significant differences in medical treatment, surgeries, or hospitalizations in the UC and in the CD groups. In summary, Mexican Americans with IBD differed significantly from other ethnic groups in the distribution of IBD subtypes, family history, and serological markers [10].

A recent large study from the United States involving 1,126 subjects with IBD, of which 830 were White, 169 were Puerto Rican Hispanics, and 127 were African Americans, reported that Hispanics were at higher risk of developing perianal disease and erythema nodosum. Among UC patients, Hispanics had more proximal disease extent. Hispanics with CD, but not UC, had lower prevalence of family history of IBD than Whites. Hispanics were more likely to have undergone bowel diversion for CD (22.4% versus 7.4%, $P = 0.001$) but had fewer total surgeries for abdominal CD than Whites. The number of surgeries for perianal CD was similar among all racial groups. At 5 years, the proportion of surgery-free CD patients was 68% for Whites and 60% for Hispanics. The median survival time free from surgery was 9.8 yr for Whites and 6.6 yr for Hispanics, with no significant increased risk of CD-related surgery for Hispanics compared to white subjects. Hispanics had a higher prevalence of surgery indicated for chronic refractory UC (85.7% versus 40.7%, $P < 0.001$). Furthermore, Hispanics had a considerably higher rate of colectomy for any indication (chronic disease, dysplasia, or fulminant colitis) than white subjects (32.3% versus 15.8%, $P < 0.01$) [7].

A comparison between our UPR IBD Registry and other Hispanic populations including Puerto Rico [7], Spain [21], Portugal [22], Huelva, Spain [23], Mexico [24], Panamá [25], and Argentina [25] is shown in Tables 3 (CD) and 4 (UC). It includes gender distribution, mean age, family history of IBD, and percent with extraintestinal manifestations. The comparison is limited by a number of small studies, the data reported for each population, and the period included in the study. As IBD incidence is considered to be increasing in Hispanics, earlier studies such as the one from Argentina and Panamá may not be representative of actual population characteristics. A systematic review of IBD in Asians, Hispanics, and African Americans published in 2009 concludes that, although the incidence is rising in Hispanics, the literature regarding IBD manifestations is very limited [26].

## 5. Conclusions

Variation in findings between different Hispanic groups may be a result of a changing epidemiology accompanying a rising incidence of IBD, with earlier studies being unable to detect an increase in family history or the true prevalence of extraintestinal manifestations, as well as an evolution in disease phenotype over time. Differences between whites and Hispanics reported in various studies are not consistent. These may be related to study design, small numbers of Hispanics in the studies, true genetic variation, and environmental influences. The limited inclusion of minorities in research studies, partly due to the low incidence of IBD at the time, but also possibly secondary to underrecruitment, poor interest in participation and referral bias, may also affect the results.

More studies are needed for a better characterization and comparison of epidemiology and clinical presentation of IBD in Hispanics. This knowledge may impact the therapeutic approach to these patients as well as the development of health care strategies to improve access and outcomes.

## Conflict of Interests

The authors have no conflict of interests to disclose.

## Authors' Contribution

All authors have had access to the data and a role in writing the paper.

## Acknowledgment

The authors wish to thank the Puerto Rico Department of Health for the support of a research fellow. The funding is institutional.

## References

[1] S. Shivananda, J. Lennard-Jones, R. Logan et al., "Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European collaborative study on inflammatory bowel disease (EC-IBD)," *Gut*, vol. 39, no. 5, pp. 690–697, 1996.

[2] A. Sonnenberg, D. J. McCarty, and S. J. Jacobsen, "Geographic variation of inflammatory bowel disease within the United States," *Gastroenterology*, vol. 100, no. 1, pp. 143–149, 1991.

[3] P. L. Lakatos, "Recent trends in the epidemiology of inflammatory bowel diseases: up or down?" *World Journal of Gastroenterology*, vol. 12, no. 38, pp. 6102–6108, 2006.

[4] E. V. Loftus Jr., "Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences," *Gastroenterology*, vol. 126, no. 6, pp. 1504–1517, 2004.

[5] M. D. Kappelman, S. L. Rifas-Shiman, K. Kleinman et al., "The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States," *Clinical Gastroenterology and Hepatology*, vol. 5, no. 12, pp. 1424–1429, 2007.

[6] A. Edouard, M. Paillaud, S. Merle, C. Orhan, and M. Chenayer-Panelatti, "Incidence of inflammatory bowel disease in the French West Indies (1997–1999)," *Gastroenterologie Clinique et Biologique*, vol. 29, no. 8-9, pp. 779–783, 2005.

[7] G. C. Nguyen, E. A. Torres, M. Regueiro et al., "Inflammatory bowel disease characteristics among African Americans, Hispanics, and non-Hispanic whites: characterization of a large North American cohort," *American Journal of Gastroenterology*, vol. 101, no. 5, pp. 1012–1023, 2006.

[8] J. H. Kurata, S. Kantor-Fish, H. Frankl, P. Godby, and C. M. Vadheim, "Crohn's disease among ethnic groups in a large health maintenance organization," *Gastroenterology*, vol. 102, no. 6, pp. 1940–1948, 1992.

[9] J. M. White, S. O'Connor, H. S. Winter et al., "Inflammatory bowel disease in African American children compared with other Racial/Ethnic groups in a multicenter registry," *Clinical Gastroenterology and Hepatology*, vol. 6, no. 12, pp. 1361–1369, 2008.

[10] D. Basu, I. Lopez, A. Kulkarni, and J. H. Sellin, "Impact of race and ethnicity on inflammatory bowel disease," *American Journal of Gastroenterology*, vol. 100, no. 10, pp. 2254–2261, 2005.

[11] W. L. Straus, G. M. Eisen, R. S. Sandler, S. C. Murray, and J. T. Sessions, "Crohn's disease: does race matter?" *American Journal of Gastroenterology*, vol. 95, no. 2, pp. 479–483, 2000.

[12] R. K. Cross, C. Jung, S. Wasan, G. Joshi, R. Sawyer, and M. C. Roghmann, "Racial differences in disease phenotypes in patients with Crohn's disease," *Inflammatory Bowel Diseases*, vol. 12, no. 3, pp. 192–198, 2006.

[13] D. G. Finlay, D. Basu, and J. H. Sellin, "Effect of race and ethnicity on perceptions of inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 12, no. 6, pp. 503–507, 2006.

[14] S. S. Mahid, A. M. Mulhall, R. D. Gholson, M. R. Eichenberger, and S. Galandiuk, "Inflammatory bowel disease and African Americans: a systematic review," *Inflammatory Bowel Diseases*, vol. 14, no. 7, pp. 960–967, 2008.

[15] A. P. Eidelwein, R. Thompson, K. Fiorino, V. Abadom, and M. Oliva-Hemker, "Disease presentation and clinical course in black and white children with inflammatory bowel disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 44, no. 5, pp. 555–560, 2007.

[16] J. F. Jackson III, T. Dhere, A. Repaka, A. Shaukat, and S. Sitaraman, "Crohn's disease in an African-American population," *American Journal of the Medical Sciences*, vol. 336, no. 5, pp. 389–392, 2008.

[17] E. A. Torres, R. De Jesús, C. M. Pérez et al., "Prevalence of inflammatory bowel disease in an insured population in Puerto Rico during 1996," *Puerto Rico health sciences journal*, vol. 22, no. 3, pp. 253–258, 2003.

[18] C. B. Appleyard, G. Hernández, and C. F. Ríos-Bedoya, "Basic epidemiology of inflammatory bowel disease in Puerto Rico," *Inflammatory Bowel Diseases*, vol. 10, no. 2, pp. 106–111, 2004.

[19] R. Vendrell, C. Perez, C. Morell et al., "Prevalence of Inflammatory Bowel Disease in an insured population in Puerto Rico during 2002–2005," in *Proceedings of the Advances in Inflammatory Bowel Disease Clinical and Research Conference*, Hollywood, Fla, USA, December 2008.

[20] J. D. Meléndez, Y. Larregui, J. M. Vázquez, V. L. Carlo, and E. A. Torres, "Medication profiles of patients in the University of Puerto Rico inflammatory bowel disease registry," *Puerto Rico Health Sciences Journal*, vol. 30, no. 1, pp. 3–8, 2011.

[21] J. Juan, "Epidemiological and clinical features of Spanish patients with Crohn's disease," *European Journal of Gastroenterology and Hepatology*, vol. 11, no. 10, pp. 1121–1127, 1999.

[22] F. Magro, F. Portela, P. Lago et al., "Crohn's disease in a Southern European country: Montreal classification and clinical activity," *Inflammatory Bowel Diseases*, vol. 15, no. 9, pp. 1343–1350, 2009.

[23] A. Garrido Serrano, M. J. Martínez, J. A. Ortega, A. Lobato, M. J. Rodríguez, and F. J. Guerrero, "Epidemiology of chronic inflammatory bowel disease in the Northern area of Huelva," *Revista Espanola de Enfermedades Digestivas*, vol. 96, no. 10, pp. 687–694, 2004.

[24] J. K. Yamamoto-Furusho, "Clinical epidemiology of ulcerative colitis in Mexico: a single hospital-based study in a 20-year period (1987–2006)," *Journal of Clinical Gastroenterology*, vol. 43, no. 3, pp. 221–224, 2009.

[25] J. A. Linares de la Cal, C. Cantón, C. Hermida, M. Pérez-Miranda, and J. Maté-Jiménez, "Tasa de incidencia estimada de Enfermedad Inflamatoria Intestinal (EII) en Argentina y Panama (1987–1993)," *Revista Espanola de Enfermedades Digestivas*, vol. 91, no. 4, pp. 277–281, 1999.

[26] J. K. Hou, H. El-Serag, and S. Thirumurthi, "Distribution and manifestations of inflammatory bowel disease in asians, hispanics, and african americans: a systematic review," *American Journal of Gastroenterology*, vol. 104, no. 8, pp. 2100–2109, 2009.

Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 505674, 11 pages
doi:10.1155/2012/505674

*Research Article*

# Assessment of the Microbiota in Microdissected Tissues of Crohn's Disease Patients

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Received 18 July 2011; Revised 5 September 2011; Accepted 14 September 2011

Academic Editor: Derek Jewell



The microbiota of the gastrointestinal tract is frequently mentioned as one of the key players in the etiopathogenesis of Crohn's disease (CD). Four hypotheses have been suggested: the single, still unknown bacterial pathogen, an abnormal overall composition of the bowel microbiota ("dysbiosis"), an abnormal immunological reaction to an essentially normally composed microbiota, and increased bacterial translocation. We propose that laser capture microdissection of selected microscopic structures, followed by broad-range 16S rRNA gene sequencing, is an excellent method to assess spatiotemporal alterations in the composition of the bowel microbiota in CD. Using this approach, we demonstrated significant changes of the composition, abundance, and location of the gut microbiome in this disease. Some of these abnormal findings persisted even after macroscopic mucosal healing. Further investigations along these lines may lead to a better understanding of the possible involvement of the bowel bacteria in the development of clinical Crohn's disease.

## 1. Introduction

Crohn's disease (CD) is characterized by chronic, segmental, transmural inflammation of the entire gastrointestinal tract. Although the exact etiology of the disease is still unclear, it is generally assumed to originate from immunologic changes induced by environmental influences in a genetically suspectible host. The first discovered CD susceptibility gene was the Caspase Recruitment Domain 15 (*CARD15*) on chromosome 16q [1, 2]. The protein product of this gene is present in monocytes, dentritic cells, Paneth cells, and intestinal epithelial cells. It recognizes a breakdown product of the bacterial cell wall, muramyl dipeptide, via its carboxyterminal leucine rich repeat region (LRR). Subsequently, it activates NF$\kappa$B. Three single nucleotide polymorphisms (SNPs) of the *CARD15* gene (Arg702Trp-SNP8, Gly908Arg-SNP12 and Leu1007fsinsC-SNP13) are associated with CD in the Caucasian population [3–7]. These 3 SNPs may interfere with the function of the LRR, potentially leading to a disturbance of the normal relationship between the human host and their bowel microbiota. Bacteria have indeed been suggested as one of the most important environmental factors in the pathogenesis of CD. Currently, four hypotheses are proposed. First, the disease may be caused by a single, still unidentified bacterial pathogen (*Mycobacterium avium* subsp. *paratuberculosis* (MAP) and adherent-invasive *Escherichia coli* are possible candidates) [8–11]. Second, the normal balance between beneficial and harmful bacterial species in the mucosa-associated microbiota may be disturbed, so-called "dysbiosis" [12–14]. Third, the mucosa may be abnormally permeable to bacteria or their products (increased bacterial translocation) [12]. Finally, the immune system may react excessively to a normally composed bowel microbiota [15]. If any of these four assumptions are true,

Table 1: Patient characteristics.

| Patient | Sex | Age | Diagnosis | *CARD15*-SNP8 | *CARD15*-SNP12 | *CARD15*-SNP13 | Procedure |
|---|---|---|---|---|---|---|---|
| 1 (*) | ♂ | 32 | CD | WT | WT | WT | Right hemicolectomy |
| 2 (*) | ♀ | 36 | CD | HE | WT | WT | Right hemicolectomy |
| 3 (*) | ♂ | 22 | CD | HE | HE | WT | Right hemicolectomy |
| 4 (*) | ♂ | 18 | CD | HO | WT | WT | Right hemicolectomy |
| 5 | ♂ | 38 | ASLC | ND | ND | ND | Colonoscopy |
| 6 (**) | ♂ | 70 | ASLC | ND | ND | ND | Colonoscopy |
| 7 | ♀ | 17 | PMC | ND | ND | ND | Colonoscopy |
| 8 (§) | ♂ | 64 | PMC | ND | ND | ND | Colonoscopy |
| 9 | ♀ | 75 | DIV | ND | ND | ND | Colonoscopy |
| 10 (§§) | ♂ | 48 | DIV | ND | ND | ND | Colonoscopy |
| 11 (*) | ♀ | 65 | AC | WT | WT | WT | Right hemicolectomy |
| 12 (*) | ♀ | 55 | AC | WT | WT | WT | Total colectomy |
| 13 (*) | ♀ | 65 | AC | HE | WT | WT | Total colectomy |

ASLC: acute self-limited colitis, PMC: pseudomembranous colitis, DIV: diverticulitis, AC: adenocarcinoma.
WT: wild type, HE: heterozygous, HO: homozygous, ND: Not determined.
SNP8: Arg702Trp, SNP12: Gly908Arg, SNP13: Leu1007fsinsC.
(*) Treated with preoperative antibiotic coverage (cefazoline 2 g IV + metronidazole 1.5 g IV at induction).
(**) Treated with amoxicillin for 2 weeks prior to colonoscopy.
(§) Clostridium difficile toxin assay positive.
(§§) Treated with amoxicillin + clavulanic acid and levoflaxacin for 10 days prior to colonoscopy.

bowel biopsies from carriers of the 3 CD-associated *CARD15* SNPs may contain unexpected bacteria or an abnormally composed microbiota in unusual locations.

Testing this hypothesis is complicated by the fact that 70 to 80% of the intestinal bacteria are currently unculturable because of fastidious or even unknown growth requirements. Culture-independent, molecular detection, and identification techniques are therefore recommended [16]. One commonly used approach is based on the structure of the 16S ribosomal RNA (rRNA) gene. This 1550 base pair long gene is found exclusively in bacteria. Its presence on the bacterial chromosome in one or more copies is necessary for normal growth and metabolism. The 16S rDNA contains both highly conserved and hypervariable (V) regions, allowing the construction of universal primers and facilitating bacterial identification up to the (sub)species level, respectively [17]. This technique has been commonly applied to faecal samples and mucosal biopsies [18]. However, a combination with laser-capture microdissection (LCM) would allow to allocate individual bacterial sequences to specific tissue compartments (e.g., the muscular layer of the bowel wall) or even microscopic lesions of interest, such as ulcers, dilated lymph vessels with surrounding inflammatory infiltrate, and granulomas. We have applied this combined approach to surgical biopsies obtained from CD patients, and we have compared our results with those obtained in (non)microdissected tissue samples from disease and healthy controls.

## 2. Materials and Methods

*2.1. Patients.* Four CD patients, 6 disease controls (acute nonchronic gastrointestinal tract inflammation), and 3 healthy controls (normal mucosa at a large distance from a nonstenosing colorectal adenocarcinoma) were included (Table 1). Genotypes for the 3 CD-associated *CARD15* SNPs were determined for the CD patients and the healthy controls using a salting out procedure starting from venous blood [19]. One to six transmural biopsies were obtained from (ileo)colectomy specimens of these patients after longitudinal opening of the bowel wall and flushing with tap water to remove residual bowel contents. For the disease controls, mucosal biopsies were obtained during colonoscopy. All biopsies were snap-frozen and stored at −80°C. One 5 $\mu$m-thick slide from each biopsy was haematoxylin-eosin stained and evaluated microscopically to confirm the diagnosis and, if applicable, to select areas suitable for LCM (Tables 2 and 3, Figure 1).

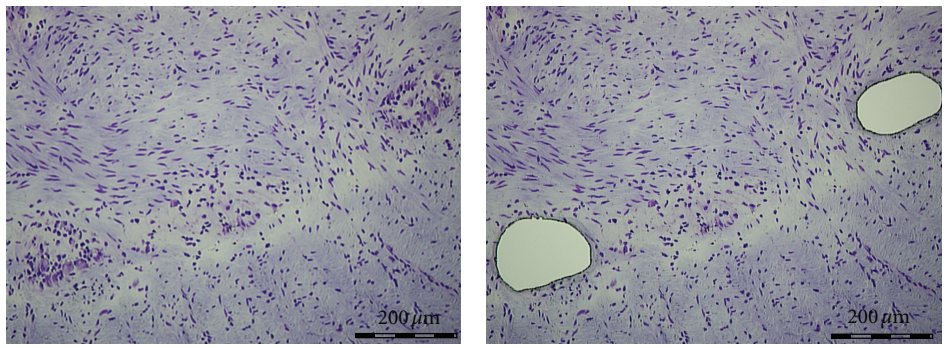
*2.2. Laser-Capture Microdissection and DNA Extraction.* For LCM, two to four 14 $\mu$m-thick tissue sections were prepared using presterilized microtome blades and UV-irradiated PALM MembraneSlides (PALM Microlaser Technologies AG, Bernried, Germany). Sections were fixed for 1 minute in 100% ethanol, dried for 15 min at 37°C, and hydrated in a decreasing concentration range of ethanol. After hydration, the slides were stained with Mayer's haematoxylin for 1 min, dehydrated in an increasing concentration range of ethanol, and air-dried under a fume hood for 5 min. The PALM Robot-MicroBeam System was used for microdissection and dissected tissue was captured in inverted Eppendorf tube caps filled with 50 $\mu$L TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Endoscopic biopsies and lymph node tissues harvested from surgical specimens were submitted to DNA extraction directly.

Tissue was digested overnight with 50 $\mu$L 2x digestion buffer (SDS 2%, 50 mM EDTA, 0.12% proteinase K) at 55°C

Table 2: Histological structures and lesions selected for LCM.

| Patient | Normal mucosa (**) | Pathological mucosa (*) (**) | Ulcer base (*) | Myenteric plexus (*) |
|---|---|---|---|---|
| 1 | IL (sample 1) | Il + COL (sample 5) | Il + COL (sample 9) | Il + COL (sample 12) |
| 2 | IL (sample 2) | Il + COL (sample 6) | NA | Il + COL (sample 13) |
| 3 | IL (sample 3) | Il + COL (sample 7) | Il + COL (sample 10) | Il + COL (sample 14) |
| 4 | IL (sample 4) | Il + COL (sample 8) | Il + COL (sample 11) | Il + COL (sample 15) |
| 11 | IL (sample 26) | NA | NA | IL (sample 30) |
| 11 | COL (sample 27) | NA | NA | COL (sample 31) |
| 12 | COL (sample 28) | NA | NA | COL (sample 32) |
| 13 | COL (sample 29) | NA | NA | COL (sample 33) |

IL: ileum; COL: colon; NA: Not applicable.
(*) IL + COL pooled per patient.
(**) The superficial half of the mucosa, the surface epithelium, and the adherent mucus were microdissected.




(a) (b)

Figure 1: Myenteric plexus before and after microdissection (Cresyl violet, ×50).

Table 3: Tissue samples which were not microdissected.

| Patient | Lymph node | Mucosal biopsy |
|---|---|---|
| 1 | IL + COL (sample 16) | NA |
| 2 | IL + COL (sample 17) | NA |
| 3 | IL + COL (sample 18) | NA |
| 4 | IL + COL (sample 19) | NA |
| 5 | NA | COL (sample 20) |
| 6 | NA | COL (sample 21) |
| 7 | NA | COL (sample 22) |
| 8 | NA | COL (sample 23) |
| 9 | NA | COL (sample 24) |
| 10 | NA | COL (sample 25) |

IL: ileum; COL: colon; NA: Not applicable.

followed by heat inactivation of proteinase K at 90°C (25 min). DNA was extracted using phenol : chloroform : isoamylacohol (25 : 24 : 1) in Heavy Phase Lock Gel tubes (Eppendorf AG, Hamburg, Germany) and dissolved in 40 $\mu$L autoclaved TE buffer for storage at −20°C. D3S3332, a human genomic DNA marker with a length of 215 base pairs (GenBank: Z38904; forward primer: 5′-GCATTT-AATGCACTAGATGCTCT-3′; reverse primer: 5′-CTTTAA-ATGCCAATTACAGTGCA-3′), was amplified from all specimens guaranteeing DNA extraction efficiency and excluding the presence of PCR inhibitors (data not shown).

Table 4: PCR primers.

| Primer | Primer sequence (5′ → 3′) |
|---|---|
| 342f | CTACGGGRSGCAGCAG |
| 515f | GTGCCAGCMGCCGCGGTAATWC |
| 1512r | TACGGYTACCTTGTTACGACTT |

M = A : C, W = A : T, R = A : G, S = C : G, Y = C : T (all 1 : 1).

*2.3. PCR Amplification of 16S rDNA.* A 998 base pair DNA fragment including the V3 to V9 regions of the 16S rRNA gene was amplified from all samples using a two rounds, heminested PCR and universal primers (1st round, primer pair: 342f/1512r; 2nd round: 515f/1512r) (Table 4). For the first round, 4 $\mu$L template DNA solution was added to 26 $\mu$L PCR mixture, obtaining final concentrations of 1x PCR buffer, 5.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 $\mu$M of each primer and 0.75 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). DNA was first denatured at 95°C for 10 minutes. Then a first PCR round of 30 cycles (denaturation 95°C, 30 sec/annealing 55°C, 30 sec/extension 72°C, 2 min)

was performed on a Tetrad-2 Thermal Cycler (Bio-Rad laboratories Inc, Calif, USA). Four $\mu$L of unpurified product of this round was used as template for the second PCR round, which also consisted of 30 cycles. Positive and negative controls (4 $\mu$L of a 1/100 diluted solution of *Escherichia coli* in LB-medium with an optical density of 0.058, and 4 $\mu$L of TE buffer, resp.) were processed simultaneously in every round. All solvents and plastic consumables were sterilized before use.

*2.4. PCR Product Analysis.* The products of the 2nd PCR round were analysed by standard agarose gel electrophoresis. Amplified DNA of the predicted size was excised and purified using the QIAquick Gel Extraction Kit (QIAGEN Benelux BV, Venlo, The Netherlands). Purified PCR products were ligated into pCR 2.1-TOPO plasmid vector using the TOPO TA Cloning Kit. Plasmids were then amplified in TOP10 One Shot Electrocompetent Cells (Invitrogen NV, Merelbeke, Belgium). Transformed cells were cultured, and amplified plasmids obtained from 8 to 48 randomly selected colonies per sample were isolated and purified using the QIAprep Turbo BioRobot Kit on a Biorobot 8000 Workstation (QIAGEN GmbH, Hilden, Germany). 16rDNA sequences were then determined by cycle sequencing using BigDye Terminator (Applied Biosystems Inc, Calif, USA) with M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) as primers. The sequences were analysed on an ABI PRISM 3730 sequencer (Applied Biosystems) and trimmed to remove vector sequence. All sequences longer than 100 nucleotides and having a greater than 98% similarity were clustered into singular operational taxonomic units (OTUs) using the ClustalW program (version 1.83, European Bioinformatics Institute), whereby OTUs are defined as groups of sequences sharing at least 98% similarity [20]. For every OTU, the sequence with the lowest number of ambiguous nucleotides was analysed with the Basic Local Alignment Search Tool (BLAST 2.2.24 release, National Center for Biotechnology Information) against the GenBank database (release 182.0). Human and vector sequences were excluded from further analysis. Possible chimeras were likewise excluded after detection using the Chimera_Check program (version 2.7) of the Ribosomal Database Project-II (RDP-II, release 9.46). An OTU was identified as a particular species when it showed at least 98% similarity with a cultured, phenotypically identified bacterial species in the databank [21]. Sequences not achieving the 98% limit were assigned to the family level of the taxonomical hierarchy proposed in *Bergey's Manual of Systematic Bacteriology* [22] using the classifier tool of the Ribosomal Database Project-II with a confidence threshold of 80%. To estimate the diversity within each sample, the cumulative number of noncontaminant bacterial families was plotted against the cumulative number of analysed bacterial sequences.

*2.5. Statistics.* Samples were compared for the abundance of various bacterial taxa using the Fisher's Exact test. $P$ values $<0.05$ were considered statistically significant.

## 3. Results

*3.1. PCR Yield and Comparison of Microdissected and Non-Microdissected Tissues.* Amplified DNA with the predicted length of 1 kb was obtained after 2 PCR rounds in all 33 tissue samples from the 13 patients. The external positive control yielded the expected product and the negative controls remained blank after 30 cycles each. Sequencing of plasmid inserts after cloning resulted in 1161 sequences longer than 100 base pairs. Overall, 410 of these (35%) originated from genuinely present bacteria belonging to 4 phyla: Proteobacteria (51%), Firmicutes (28%), Actinobacteria (12%), and Bacteroidetes (9%). These 410 sequences could be further classified into 20 different families, and 147 sequences (36%) were identified unambiguously at the species level: *Escherichia coli* (17.5%), *Kocuria rosea* (6.6%), *Propionibacterium acnes* (2.9%), *Enterococcus faecalis* (2.2%), *Lactobacillus rhamnosus* (1.7%), *Streptococcus mitis* (1.7%), *Eubacterium rectale* (1.5%), *Bacteroides fragilis* (0.7%), *Clostridium nexile* (0.2%), *Bacteroides eggerthii* (0.2%), and *Bacteroides uniformis* (0.2%) (Table 5). No *Faecalibacterium prausnitzii* was identified on species level in the CD cases. Of the 751 remaining sequences, 55 were due to primer misalignment, 3 were likely chimeric, 187 originated from human DNA, and 506 were derived from probable contaminants belonging to the classes of the Alpha- and Betaproteobacteria and the families Pseudomonadaceae and Staphylococcaceae. Amplified contaminating DNA was significantly more commonly encountered in PCR products from microdissected versus non-microdissected tissues (71.0% and 1.4% of all bacterial sequences, resp.). The same applied to the proportion of sample-derived sequences of human instead of bacterial origin (47.0% and 2.4% in microdissected versus non-microdissected tissues, resp.) (Fisher's Exact test: $P < 0.001$ in both occasions). The cumulative numbers of detected genuinely present bacterial families plotted against the total numbers of analysed bacterial sequences revealed increasing slopes for most microdissected samples, while reaching asymptotic values for the non-microdissected tissues (Figure 2).

*3.2. Comparison of the Microbiota in Crohn's Disease and Controls.* Microdissected normal ileal mucosa from a healthy control yielded only 1 sequence, which was derived from the family of the Enterobacteriaceae. Normal colon mucosa yielded more sequences, pointing to a more abundant adherent microbiota which also contained strictly anaerobic bacteria (Bacteroidetes). Microbiota diversity was higher in the disease controls, with variation even between cases with an identical diagnosis. For example, one case of acute self-limited colitis showed a preponderance of sequences derived from Enterobacteriaceae while no potential pathogens were detected in the other patient's sample. Similarly, in 1 case of pseudomembranous colitis (PMC) all sequences were derived with certainty from *Lactobacillus rhamnosus*, while in the second case the microbiota was diverse but did not contain *Clostridium difficile*. One diverticulitis sample was completely colonized by Enterobacteriaceae, while the other showed a predominantly anaerobic adherent microbiota.

Table 5: Distribution of bacterial taxa over the 33 tissue samples (*) (**).

| Identification | CD | | | | Disease controls | | | | | | | Healthy controls | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IL, normal mucosa (samples 1–4) | IL+COL, pathological mucosa (samples 5–8) | IL+COL, ulcer (samples 9–11) | IL+COL, myenteric plexus (samples 12–15) | lymph node (samples 16–19) | COL, ASLC (sample 20) | COL, ASLC (sample 21) | COL, PMC (sample 22) | COL, PMC (sample 23) | COL, DIV (sample 24) | COL, DIV (sample 25) | IL, normal mucosa (sample 26) | COL, normal mucosa (samples 17–29) | IL, myenteric plexus (sample 30) | COL, myenteric plexus (samples 31–33) |
| Total number of sequences (domain bacteria) | 18 | 22 | 52 | 45 | 79 | 15 | 14 | 12 | 7 | 40 | 38 | 1 | 24 | 35 | 8 |
| Phylum XII. Proteobacteria | | | | | | | | | | | | | | | |
| Class III. Gammaproteobacteria | | | | | | | | | | | | | | | |
| Order III. Xanthomonadales | | | | | | | | | | | | | | | |
| Family I. Xanthomonadaceae | | | | 11 | | | | | | | | | | | |
| Order VI. Legionellales | | | | | | | | | | | | | | | |
| Family I. Legionellaceae | | | | 51 | | | | | | | | | | | |
| Order IX. Pseudomonadales | | | | | | | | | | | | | | | |
| Family II. Moraxellaceae | | | | 18 | | | | | | | | | | | |
| Order XII. Aeromonadales | | | | | | | | | | | | | | | |
| Family II. Succinivibrionaceae | | | | | 1 | | | | | | | | | | |
| Order XIII. Enterobacteriales | | | | | | | | | | | | | | | |
| Family I. Enterobacteriaceae | 56 | 68 | 67 | | 15 | 53 | | | | 5 | 32 | 100 | | | |
| *Escherichia coli* | | | | | 43 | | | | | 95 | | | | | |
| Order XIV. Pasteurellales | | | | | | | | | | | | | | | |
| Family I. Pasteurellaceae | | | | | 1 | | | | | | 8 | | | | |
| Class V. Epsilonproteobacteria | | | | | | | | | | | | | | | |
| Order I. Campylobacterales | | | | | | | | | | | | | | | |
| Family I. Campylobacteraceae | | | | | | | | 8 | | | | | | | |
| Phylum XIII. Firmicutes | | | | | | | | | | | | | | | |
| Class I. Clostridia | | | | | | | | | | | | | | | |
| Order I. Clostridiales | | | | | | | | | | | | | | | |
| Family I. Clostridiaceae | 28 | | | | 13 | | | 8 | | | 8 | | | | |
| *Clostridium nexile* | | | | | 1 | | | | | | | | | | |
| Family II. Lachnospiraceae | 11 | 5 | | | 5 | | | | | | 18 | | | | |
| Family IV. Eubacteriaceae | | | | | 1 | | | | | | 8 | | | | |
| *Eubacterium rectale* | | | | | | | | | | | 16 | | | | |
| Family VII. Acidaminococcaceae | | | 33 | | | | | | | | 3 | | | | |
| Class III. Bacilli | | | | | | | | | | | | | | | |
| Order I. Bacillales | | | | | | | | | | | | | | | |
| Family I. Bacillaceae | | | | | | | 7 | | | | | | | | 100 |
| Order II. Lactobacillales | | | | | | | | | | | | | | | |
| Family I. Lactobacillaceae | | | | | | | 7 | 8 | | | | | | | |
| *Lactobacillus rhamnosus* | | | | | | | | | 100 | | | | | | |
| Family IV. Enterococcaceae | | | | | | | 21 | | | | | | | | |
| *Enterococcus faecalis* | | | | | | | 64 | | | | | | | | |

Table 5: Continued.

| Identification | CD | | | | Disease controls | | | | | | | Healthy controls | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IL, normal mucosa (samples 1–4) | IL+COL, pathological mucosa (samples 5–8) | IL+COL, ulcer (samples 9–11) | IL+COL, myenteric plexus (samples 12–15) | lymph node (samples 16–19) | COL, ASLC (sample 20) | COL, ASLC (sample 21) | COL, PMC (sample 22) | COL, PMC (sample 23) | COL, DIV (sample 24) | COL, DIV (sample 25) | IL, normal mucosa (sample 26) | COL, normal mucosa (samples 17–29) | IL, myenteric plexus (sample 30) | COL, myenteric plexus (samples 31–33) |
| Total number of sequences (domain bacteria) | 18 | 22 | 52 | 45 | 79 | 15 | 14 | 12 | 7 | 40 | 38 | 1 | 24 | 35 | 8 |
| Family VI. Streptococcaceae | | | | 4 | | | | 8 | | | 3 | | 50 | | |
| *Streptococcus mitis* | | | | | | | | 58 | | | | | | | |
| Phylum XIV. Actinobacteria | | | | | | | | | | | | | | | |
| Class I. Actinobacteria | | | | | | | | | | | | | | | |
| Order I. Actinomycetales | | | | | | | | | | | | | | | |
| Family I. Micrococcaceae | | | | | | | | | | | | | | 9 | |
| *Kocuria rosea* | | | | 2 | | | | | | | | | | 74 | |
| Family I. Propionibacteriaceae | | | | 13 | | | | | | | | | | 3 | |
| *Propionibacterium acnes* | 6 | 27 | | | | | | | | | | | | 14 | |
| Phylum XX. Bacteroidetes | | | | | | | | | | | | | | | |
| Class I. Bacteroidetes | | | | | | | | | | | | | | | |
| Order I. Bacteroidales | | | | | | | | | | | | | | | |
| Family I. Bacteroidaceae | | | | | 14 | 7 | | | | | 3 | | 50 | | |
| *Bacteroides fragilis* | | | | | 4 | | | | | | | | | | |
| *Bacteroides eggerthii* | | | | | | | | 8 | | | | | | | |
| *Bacteroides uniformis* | | | | | | | | | | | 3 | | | | |
| Family III. Porphyromonadaceae | | | | | 1 | | | | | | | | | | |
| Family IV. Prevotellaceae | | | | | | 40 | | | | | | | | | |

(*) Numbers indicated per taxon are percentages of the total number of sequences indicated in the first row.
(**) Empty cells indicate value of 0%.

Overall however, sequence analysis of the PCR products indicated that there might be a shift towards a nonstrictly anaerobic microbiota in disease controls versus healthy controls (Fisher's Exact test: $P = 0.0243$).

Histological normal microdissected ileal mucosa in Crohn's disease contained a fairly abundant, mixed aerobic—anaerobic microbiota. With ulceration, there was an increase in both the number of detectable bacterial sequences and the fraction of nonstrictly anaerobes (67% versus 62% in normal mucosa). This underrepresentation of anaerobes in ulcers was retained and seemed even more accentuated in so-called pathological mucosa, which shows macroscopic healing but is histologically still abnormal (Fisher's Exact test: $P = 0.0138$). An interesting feature of the myenteric plexus in Crohn's disease samples was the detection of DNA from a single bacterial species belonging to the family Legionellaceae. In this study, this biological signal was picked up exclusively in CD patients carrying at least 1 copy of either SNP8 or SNP12 of the *CARD15* gene (Table 1). No Legionellaceae were detected in controls or in the CD patient without CD-associated *CARD15* mutations. Tissue samples from ileocolonic lymph nodes in CD patients contained bacterial DNA, compatible with bacterial translocation. The translocating microbiota was mixed, with a higher proportion of strict anaerobes than in the bowel wall proper (39% versus 18%, Fisher's Exact test: $P = 0.0007$).

## 4. Discussion

The role of the gut microbiota in the etiopathogenesis of Crohn's disease is a hotly debated topic, and many experimental protocols have been devised to investigate this

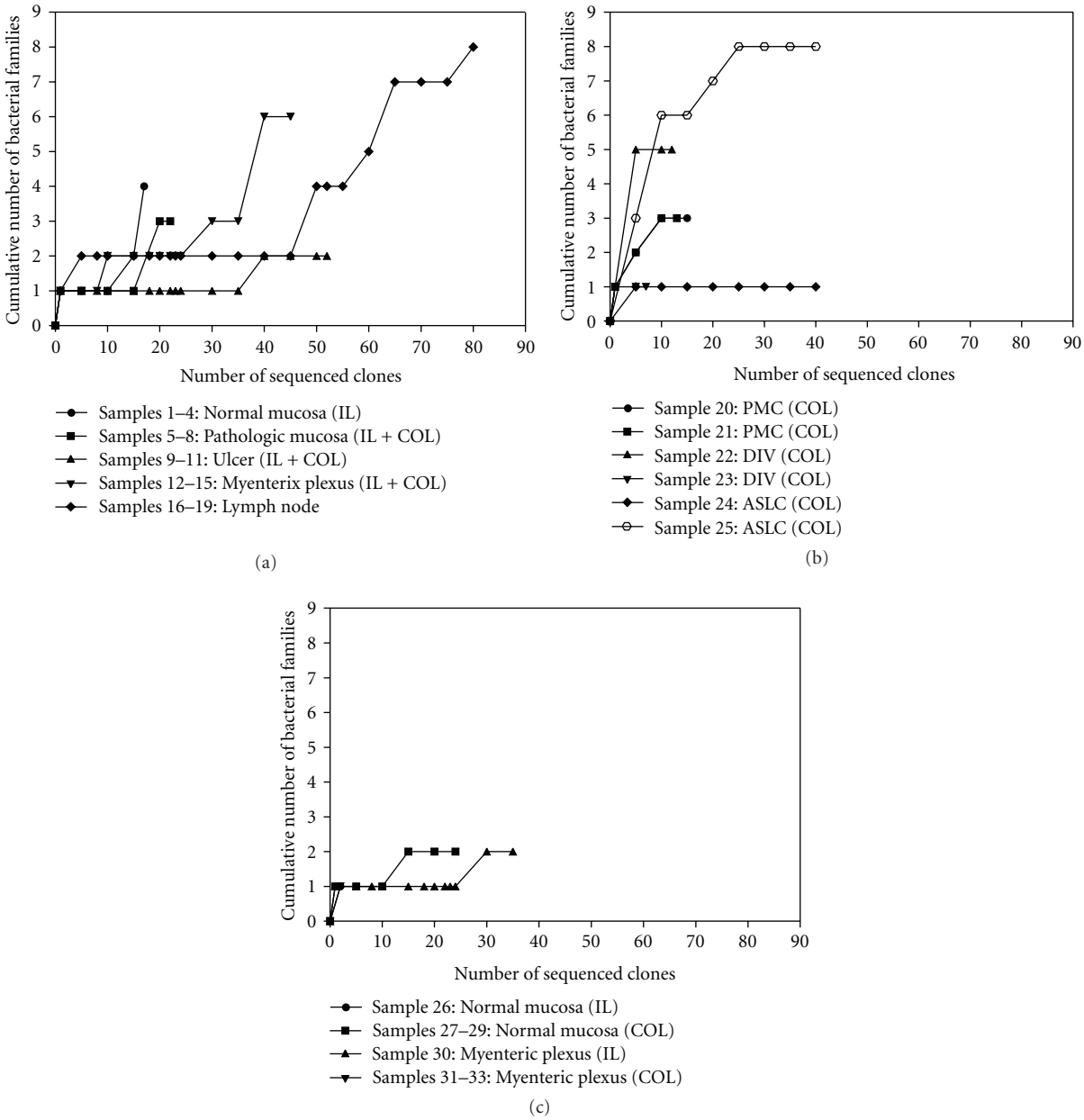



Figure 2: Plots of the cumulative numbers of detected genuinely present bacterial families versus the total number of bacterial sequences analysed for CD patients (a), disease controls (b), and healthy controls (c). ASLC: acute self-limited colitis, PMC: pseudomembranous colitis, DIV: diverticulitis, AC: adenocarcinoma. IL: ileum, COL: colon.

problem. In this study, we applied broad-range 16S rDNA PCR using universal primers, followed by sequencing of PCR products and bacterial identification based on the obtained sequences, an approach which has been used previously for faecal samples and mucosal biopsies [18]. The novelty of our approach is its application to microdissected normal tissue structures or CD-associated lesions such as ulcerations and architecturally abnormal, chronically inflamed mucosa. Theoretically, this combination would allow the identification of single bacterial species in well-defined locations, which may facilitate the detection of those microorganisms which are most likely involved in CD pathogenesis. To this end, results from CD cases were also compared with those obtained in microdissected and non-microdissected disease and healthy controls.

Laser capture microdissection was performed on fresh-frozen transmural bowel biopsies obtained from surgical samples. As part of the standard protocol, patients underwent gut lavage prior to surgery, and broad-spectrum preoperative antibiotics were administered to diminish the risk of sepsis. Also, the surgical specimens were flushed with tap water to remove residual bowel contents before transmural biopsies were obtained. These interventions will certainly lower the overall amount of mucosa-adherent bacteria, but

will not necessarily eliminate them or significantly distort the original composition of the microbiota [23].

In this study, we microdissected 4 different bowel wall regions which were chosen based upon their expected informative value with regard to disease pathogenesis. The first area is the mucosa, which was selected since the highest number of bacteria is expected in this location [24]. Additionally, in CD a distinction can be made between structurally normal and pathological mucosa, which may be macroscopically intact but histologically abnormal. Ulcer beds are another lesion of interest, given their observable histological progression, frequency, and severity in this disease. The myenteric plexus is a well-recognizable histological landmark in bowel resections and may thus serve as a tissue sample suitable for the study of bacteria or bacterial products in passage through the bowel wall. Another important though not universal or entirely specific feature of CD is epithelioid granulomas. Two previous studies applying LCM and molecular bacteriological detection methods have demonstrated that these lesions can contain DNA from MAP and *Escherichia coli* [25, 26]. Another study from our group, using LCM and a universal bacterial PCR, suggested that the microbiota contained in granulomas may be even more diverse [27]. Based on these data, we now hypothesize that granulomas in Crohn's disease may act as areas of containment for all kinds of translocating bacteria. Testing this hypothesis would require a large-scale investigation of granuloma tissue obtained from surgical samples of patients with various genetic backgrounds, disease locations, and phenotypes. This is obviously beyond the scope of the current study. As an alternative, we chose to examine mesenteric lymph nodes, which also serve as a collector and filter for all the drained lymphatic fluid from both normal and pathological bowel segments.

Almost half of the sequences which we obtained in this study were derived from Alpha- and Betaproteobacteria, Pseudomonadaceae, and Staphylococcaceae. According to most previous descriptions, these taxa are distinctly uncommon in both the normal faecal and mucosa-associated bowel microbiota. We therefore assume that they are contaminants, which may have been present in the intact form or only as residual DNA either in the tap water used to flush the specimens on the surface of presterilized gloves and consumables or even in ultrapure water used for molecular tests [28].

We first investigated the normal mucosa-adherent microbiota in the ileum and colon of "healthy" controls, which we defined as tissue samples taken from macroscopically normal bowel at a large distance from the tumor in patients operated upon for colorectal adenocarcinoma. Since these tumors were nonstricturing and since preoperative gut lavage was possible in each patient, we assume that the presence of a tumor did not influence the composition of the mucosal microbiota in our samples. Results were as expected with regard to both the quantitative and qualitative aspect: the ileum was scarcely populated while colon mucosa contained a more abundant microbiota with a high fraction of strictly anaerobic bacteria.

Bacterial infections are characterized by microbial population dynamics changing over time, which may moreover be dependent on extraneous variables such as the administration of anti- and possibly probiotics. Therefore we investigated 3 types of disease controls, that is, cases of acute self-limited colitis (a pathological entity which in some cases has an infectious origin), pseudomembranous colitis, and diverticulitis (which has also been associated with changes in the composition of the faecal microbiota) [29–31]. Our results illustrate the disturbance of the normal mucosal population patterns in these conditions. We did not specifically identify *Clostridium difficile* in the cases of PMC. However, it should be noted that the standard toxin assays are performed on faecal samples, and that neither the classic assay nor the PCR for the *tcdB* toxin gene is entirely specific when compared with the gold standard of sensitive bacterial culture [32, 33]. It can thus be expected that in some toxin-positive cases, *C. difficile* cannot be isolated from stool samples even with the best available culture methods. A fortiori this would apply to mucosal biopsies which provide a much smaller amount of starting material. Moreover it is not known whether *C. difficile* in pseudomembranous colitis is mucosa-associated or dwells in the bowel lumen or in the pseudomembranes. It is indeed difficult to pinpoint the presence of specific bacterial pathogens, for which the spatial and temporal distribution during the course of the infectious disease is a priori unknown. Our results may also have been influenced by the previous administration of various courses of antibiotics or possibly even probiotics (*Lactobacillus rhamnosus*) in some patients. Investigators of the microbiota in Crohn's disease should therefore always take into account a history of prior antibiotic therapy or administration of probiotics in their study subjects.

The only objective difference between histological normal ileal mucosa in Crohn's disease cases and the healthy control patient was the greater ease with which DNA from genuinely present bacteria was detected in the former. We can thus speculate that the absolute number of mucosa-adherent bacteria is larger in CD patients compared to controls, which fits with previous reports in the literature [34, 35]. We may further assume that the composition of the mucosal microbiota will show additional changes with the development of ulcerations. There are at least 2 possibilities: some bacterial variants may induce ulcers (e.g., the adherent-invasive subtype of *Escherichia coli*), or the presence of an ulcer slough and underlying granulation tissue may confer a selective growth advantage to particular bacterial taxa. In this study, no specific pathogen was detected in microdissected ulcer beds in Crohn's disease. There were however indications that these lesions may show localized bacterial overgrowth, with shifts in the composition of the microbiota possibly associated with an altered physicochemical milieu.

Previous studies on faecal samples have demonstrated an abnormally high biodiversity of the microbiota in CD patients in remission. Some harmful bacteria are increased (e.g., *Bacteroides fragilis,* an opportunistic pathogen) and the amount of protective, butyrate producing Firmicutes (e.g., *Faecalibacterium prausnitzii*) is often diminished in these patients [16, 24, 36, 37]. This dysbiosis is apparently independent of disease location, presence of anatomical abnormalities secondary to inflammation or scarring, treatment with sulphasalazine or corticosteroids and even surgery.

Ileocolonoscopy in these patients often shows mucosal healing, with residual signs of inactive chronic inflammation on biopsy. Similar histological features can be seen in some macroscopically normal bowel segments in surgical specimens of patients operated upon for complicated Crohn's disease. We therefore investigated whether the mucosa-associated microbiota is also persistently altered in such areas of microscopically "pathological mucosa." Our results show that this is indeed the case, with a significant increase in nonstrictly anaerobic taxa when compared with structurally normal mucosa.

It has been previously proposed that viable bacteria may be capable of crossing the intestinal mucosal barrier and of trafficking to extraintestinal sites such as the mesenteric lymph nodes, the systemic circulation, the liver and the spleen [38, 39]. This "bacterial translocation" may be a phenomenon that occurs in healthy individuals without deleterious consequences. Translocation of endotoxins from viable or dead bacteria in very small amounts probably constitutes a physiologically important boost to the reticulo-endothelial system, especially to the Kupffer cell in the liver. The baseline rate of translocation in human studies is 5–10% [39, 40]. Previous studies also indicate that Enterobacteriaceae translocate much more efficiently than other bacteria (especially obligate anaerobes) [39]. Bacterial translocation would be further promoted by 3 major conditions: intestinal bacterial overgrowth, deficiencies in host immune defenses, and increased mucosal permeability or major structural damage to the intestinal mucosal barrier [38]. Under these conditions, which may all be present in active Crohn's disease, one may expect a significant increase in the abundance of potentially pathogenic bacteria or bacterial products in the deeper layers of the bowel wall or in the surrounding lymph nodes. We tested this hypothesis for 2 anatomical compartments: the myenteric plexus as a surrogate for bacterial trafficking within the bowel wall and mesenteric lymph node tissue as the ideal locus to detect an even more advanced and potentially more dangerous form of bacterial translocation.

The main difference between the myenteric plexus samples of CD patients and healthy controls was the presence of a particular Legionella species in some of the former, more specifically only in our CD patients carrying at least one copy of either SNP8 or SNP12 of the *CARD15* gene. The relevant PCR product could not be identified at the species level, but the closest relative was *Legionella lytica*. Legionellaceae are gram-negative bacteria, which are able to reproduce at temperatures between 25°C and 43°C and survive in temperatures of up to 55–60°C. They are therefore ubiquitous in natural and artificial aquatic environments [41]. Since this sequence was only detected in CD patients and not in controls, the corresponding Legionella species may fit in the hypothesis of the persistent, still-unknown, pathogen. It could then be placed next to *Mycobacterium avium* subsp. *paratuberculosis* and adherent-invasive *Escherichia coli* which were also initially detected in very few CD patients [8–11]. The second reason why this finding may be relevant to the study of the etiopathogenesis of Crohn's disease is the known association of myenteric plexitis in the proximal margin of surgical specimens with early clinical and histological disease recurrence in the neoterminal ileum [42, 43].

Finally, our results on mesenteric lymph node tissues confirm the occurrence and the potential extent of bacterial translocation in Crohn's disease. The biological diversity of the translocating microbiota was large, with sequences from 8 different families being detected. *Escherichia coli* was the single most frequently detected species. Since the ratio of facultative over strict anaerobic bacteria was 1.47, facultative anaerobes may translocate more efficiently at larger distances.

## 5. Conclusion

In this study, we investigated alterations in the mucosa-associated and translocating bowel microbiota in Crohn's disease patients, disease controls, and healthy controls. We used a new approach consisting of laser capture microdissection of selected microscopic structures, followed by broad-range 16S rDNA PCR with universal primers. Our findings in healthy and disease controls are compatible with previous culture-based and molecular studies. They underline the significance of biopsy location and prior or concurrent antimicrobial therapy for a correct interpretation of the composition of the mucosa-associated bowel microbiota. Our results in Crohn's disease point to important spatiotemporal alterations of the gut microbiome in this condition. The composition and abundance of the mucosa-associated microbiota changes with the presence of mucosal defects and seems to remain abnormal upon macroscopic healing. There are also indications of increased bacterial translocation, possibly by uncommon bacterial species which may find a new ecological niche in the inflamed bowel wall. Further investigations using the combination of LCM and universal 16S rDNA PCR may lead to a better understanding of the role of the bowel microbiota in the etiopathogenesis of Crohn's disease.

## Acknowledgment

The authors would like to thank Dr. K. Geboes (em.) for his advice during the preparation of the paper.

## References

[1] J. P. Hugot, M. Chamaillard, H. Zouali et al., "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease," *Nature*, vol. 411, no. 6837, pp. 599–603, 2001.

[2] Y. Ogura, D. K. Bonen, N. Inohara et al., "A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease," *Nature*, vol. 411, no. 6837, pp. 603–606, 2001.

[3] D. A. van Heel, K. A. Hunt, K. King et al., "Detection of muramyl dipeptide-sensing pathway defects in patients with Crohn's disease," *Inflammatory Bowel Diseases*, vol. 12, no. 7, pp. 598–605, 2006.

[4] D. K. Bonen and J. H. Cho, "The genetics of inflammatory bowel disease," *Gastroenterology*, vol. 124, no. 2, pp. 521–536, 2003.

[5] A. Lecat, J. Piette, and S. Legrand-Poels, "The protein Nod2: an innate receptor more complex than previously assumed," *Biochemical Pharmacology*, vol. 80, no. 12, pp. 2021–2031, 2010.

[6] J. G. Magalhaes, M. T. Sorbara, S. E. Girardin, and D. J. Philpott, "What is new with Nods?" *Current Opinion in Immunology*, pp. 29–34, 2011.

[7] J. Van Limbergen, D. C. Wilson, and J. Satsangi, "The genetics of Crohn's disease," *Annual Review of Genomics and Human Genetics*, vol. 10, pp. 89–116, 2009.

[8] J. L. Mendoza, A. San-Pedro, E. Culebras et al., "High prevalence of viable mycobacterium avium subspecies paratuberculosis in crohn's disease," *World Journal of Gastroenterology*, vol. 16, no. 36, pp. 4558–4563, 2010.

[9] S. Toracchio, H. M. T. El-Zimaity, C. Urmacher, S. Katz, and D. Y. Graham, "Mycobacterium avium subspecies paratuberculosis and Crohn's disease granulomas," *Scandinavian Journal of Gastroenterology*, vol. 43, no. 9, pp. 1108–1111, 2008.

[10] E. S. Pierce, "Where are all the Mycobacterium avium subspecies paratuberculosis in patients with crohn's disease?" *PLoS Pathogens*, vol. 5, no. 3, Article ID e1000234, 2009.

[11] M. Martinez-Medina, A. Mora, M. Blanco et al., "Similarity and divergence among adherent-invasive Escherichia coli and extraintestinal pathogenic E. coli strains," *Journal of Clinical Microbiology*, vol. 47, no. 12, pp. 3968–3979, 2009.

[12] G. De Hertogh, J. Aerssens, K. P. Geboes, and K. P. Geboes, "Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease," *World Journal of Gastroenterology*, vol. 14, no. 6, pp. 845–852, 2008.

[13] C. P. Tamboli, C. Neut, P. Desreumaux, and J. F. Colombel, "Dysbiosis in inflammatory bowel disease," *Gut*, vol. 53, no. 1, pp. 1–4, 2004.

[14] M. Joossens, G. Huys, M. Cnockaert et al., "Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives," *Gut*, vol. 60, pp. 631–637, 2011.

[15] A. Swidsinski, V. Loening-Baucke, and A. Herber, "Mucosal flora in Crohn's disease and ulcerative colitis—an overview," *Journal of Physiology and Pharmacology*, vol. 60, supplement 6, pp. 61–71, 2009.

[16] P. D. Scanlan, F. Shanahan, C. O'Mahony, and J. R. Marchesi, "Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease," *Journal of Clinical Microbiology*, vol. 44, no. 11, pp. 3980–3988, 2006.

[17] J. E. Clarridge III, "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases," *Clinical Microbiology Reviews*, vol. 17, no. 4, pp. 840–862, 2004.

[18] R. B. Sartor, "Microbial influences in inflammatory bowel diseases," *Gastroenterology*, vol. 134, no. 2, pp. 577–594, 2008.

[19] N. Esters, M. Pierik, K. Van Steen et al., "Transmission of CARD15 (NOD2) variants within families of patients with inflammatory bowel disease," *The American Journal of Gastroenterology*, vol. 99, no. 2, pp. 299–305, 2004.

[20] R. Rossello-Mora and R. Amann, "The species concept for prokaryotes," *FEMS Microbiology Reviews*, vol. 25, no. 1, pp. 39–67, 2001.

[21] E. Stackebrandt and B. M. Goebel, "Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology," *International Journal of Systematic Bacteriology*, vol. 44, no. 4, pp. 846–849, 1994.

[22] G. Garrity, J. Bell, and T. Lilburn, "Taxonomic outline of the prokaryotes," in *Bergey's Manual of Systemic Bacteriology*, G. Garrity, Ed., pp. 1–401, Springer, New York, NY, USA, 2004.

[23] H. Fujita, Y. Eishi, I. Ishige et al., "Quantitative analysis of bacterial DNA from Mycobacteria spp., Bacteroides vulgatus, and Escherichia coli in tissue samples from patients with inflammatory bowel diseases," *Journal of Gastroenterology*, vol. 37, no. 7, pp. 509–516, 2002.

[24] P. Marteau, "Bacterial flora in inflammatory bowel disease," *Digestive Diseases*, vol. 27, supplement 1, pp. 99–103, 2009.

[25] P. Ryan, M. W. Bennett, S. Aarons et al., "PCR detection of Mycobacterium paratuberculosis in Crohn's disease granulomas isolated by laser capture microdissection," *Gut*, vol. 51, no. 5, pp. 665–670, 2002.

[26] P. Ryan, R. G. Kelly, G. Lee et al., "Bacterial DNA within granulomas of patients with Crohn's disease—detection by laser capture microdissection and PCR," *The American Journal of Gastroenterology*, vol. 99, no. 8, pp. 1539–1543, 2004.

[27] G. de Hertogh, J. Aerssens, R. de Hoogt et al., "Validation of 16S rDNA sequencing in microdissected bowel/biopsies from Crohn's disease patients to assess bacterial flora diversity," *Journal of Pathology*, vol. 209, no. 4, pp. 532–539, 2006.

[28] L. A. Kulakov, M. B. McAlister, K. L. Ogden, M. J. Larkin, and J. F. O'Hanlon, "Analysis of bacteria contaminating ultrapure water in industrial systems," *Applied and Environmental Microbiology*, vol. 68, no. 4, pp. 1548–1555, 2002.

[29] N. B. Kumar, T. T. Nostrant, and H. D. Appelman, "The histopathologic spectrum of acute self-limited colitis (acute infectious-type colitis)," *The American Journal of Surgical Pathology*, vol. 6, no. 6, pp. 523–529, 1982.

[30] T. Gouliouris, N. M. Brown, and S. H. Aliyu, "Prevention and treatment of Clostridium difficile infection," *Clinical Medicine*, vol. 11, pp. 75–79, 2011.

[31] A. Tursi and S. Papagrigoriadis, "Review article: the current and evolving treatment of colonic diverticular disease," *Alimentary Pharmacology and Therapeutics*, vol. 30, no. 6, pp. 532–546, 2009.

[32] M. Kawada, M. Annaka, H. Kato et al., "Evaluation of a simultaneous detection kit for the glutamate dehydrogenase antigen and toxin A/B in feces for diagnosis of *Clostridium difficile* infection," *Journal of Infection and Chemotherapy*. In press.

[33] F. Barbut, M. Monot, A. Rousseau et al., "Rapid diagnosis of Clostridium difficile infection by multiplex real-time PCR," *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 30, no. 10, pp. 1279–1285, 2011.

[34] M. Martinez-Medina, X. Aldeguer, F. Gonzalez-Huix, D. Acero, and L. J. Garcia-Gil, "Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis," *Inflammatory Bowel Diseases*, vol. 12, no. 12, pp. 1136–1145, 2006.

[35] A. Swidsinski, V. Loening-Baucke, M. Vaneechoutte, and Y. Doerffel, "Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora," *Inflammatory Bowel Diseases*, vol. 14, no. 2, pp. 147–161, 2008.

[36] A. Swidsinski, A. Ladhoff, A. Pernthaler et al., "Mucosal flora in inflammatory bowel disease," *Gastroenterology*, vol. 122, no. 1, pp. 44–54, 2002.

[37] H. Sokol, P. Seksik, J. P. Furet et al., "Low counts of faecalibacterium prausnitzii in colitis microbiota," *Inflammatory Bowel Diseases*, vol. 15, no. 8, pp. 1183–1189, 2009.

[38] R. D. Berg, "Bacterial translocation from the gastrointestinal tract," *Trends in Microbiology*, vol. 3, no. 4, pp. 149–154, 1995.

[39] F. Guarner and J. R. Malagelada, "Gut flora in health and disease," *The Lancet*, vol. 361, no. 9356, pp. 512–519, 2003.

[40] S. Balzan, C. de Almeida Quadros, R. de Cleva, B. Zilberstein, and I. Cecconello, "Bacterial translocation: overview of mechanisms and clinical impact," *Journal of Gastroenterology and Hepatology*, vol. 22, no. 4, pp. 464–471, 2007.

[41] S. W. Huang, B. M. Hsu, S. F. Wu et al., "Water quality parameters associated with prevalence of Legionella in hot spring facility water bodies," *Water Research*, vol. 44, no. 16, pp. 4805–4811, 2010.

[42] M. Ferrante, G. de Hertogh, T. Hlavaty et al., "The value of myenteric plexitis to predict early postoperative Crohn's disease recurrence," *Gastroenterology*, vol. 130, no. 6, pp. 1595–1606, 2006.

[43] H. Sokol, V. Polin, A. Lavergne-Slove et al., "Plexitis as a predictive factor of early postoperative clinical recurrence in Crohn's disease," *Gut*, vol. 58, no. 9, pp. 1218–1225, 2009.