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ORIGINAL ARTICLE

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ORIGINAL ARTICLE

Basic Study

Pentadecapeptide BPC 157 resolves suprahepatic occlusion of the inferior caval vein, Budd-Chiari syndrome model in rats

Slaven Gojkovic, Ivan Krezic, Borna Vrdoljak, Dominik Malekinusic, Ivan Barisic, Andreja Petrovic, Katarina Horvat Pavlov, Marijan Kolovrat, Antonija Duzel, Mario Knezevic, Katarina Kasnik Kovac, Domagoj Drmic, Lovorka Batelja Vuletic, Antonio Kokot, Alenka Boban Blagaic, Sven Seiwerth, Predrag Sikiric

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Abstract

BACKGROUND

Recently, as a possible therapy resolving solution, pentadecapeptide BPC 157 therapy, has been used in alleviating various vascular occlusion disturbances. BPC 157 was previously reviewed as novel mediator of Robert cytoprotection and endothelium protection in the stomach, and gut-brain axis, beneficial therapy in gastrointestinal tract, with particular reference to vascular recruitment, ulcerative colitis and tumor cachexia, and other tissues healing. Here we raised new hypothesis about BPC 157 therapy in the Budd-Chiari syndrome in rats, rapid bypassing of the suprahepatic inferior caval vein occlusion, and rats recovery with the active and effective pharmacotherapy treatment.

AIM

To investigate Budd-Chiari syndrome model (inferior caval vein suprahepatic occlusion) resolution, since BPC 157 resolves various rat vascular occlusion.

METHODS

We assessed the activated bypassing pathways between the inferior and superior caval veins and portocaval shunt, counteracted caval/portal hypertension, aortal hypotension, venous/arterial thrombosis, electrocardiogram disturbances, liver and gastrointestinal lesions (*i.e.*, stomach and duodenum hemorrhages, in particular, congestion). Rats with suprahepatic occlusion of the inferior vena cava by ligation were medicated at 1 min, 15 min, 24 h, or 48 h post-ligation. Medication consisted of 10 μg/kg BPC 157, 10 ng BPC 157 or 5 mL/kg saline, administered once as an abdominal bath or intragastric application. Gross and microscopic observations were made, in addition to assessments of electrical

tools; Gojkovic S, Drmic D, Seiwerth S and Sikiric P wrote the manuscript.

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activity of the heart (electrocardiogram), portal and caval hypertension, aortal hypotension, thrombosis, hepatomegaly, splenomegaly and venography. Furthermore, levels of nitric oxide, malondialdehyde in the liver and serum enzymes were determined.

RESULTS
BPC 157 counteracted increased P wave amplitude, tachycardia and ST-elevation, *i.e.*, right heart failure from acute thrombotic coronary occlusion. The bypassing pathway of the inferior vena cava-azygos (hemiazygos) vein-superior vena cava and portocaval shunt occurred rapidly. Even with severe caval portal hypertension, BPC 157 antagonized portal and caval hypertension and aortal hypotension, and also reduced refractory ascites. Thrombosis of portal vein tributaries, inferior vena cava, and hepatic and coronary arteries was attenuated. In addition, there was reduced pathology of the lungs (severe capillary congestion) and liver (dilated central veins and terminal portal venules), decreased intestine hemorrhagic lesions (substantial capillary congestion, submucosal edema and architecture loss), and increased liver and spleen weight. During the period of ligation, nitric oxide- and malondialdehyde-levels in the liver remained within normal healthy values, and increases in serum enzymes were markedly reduced.

CONCLUSION
BPC 157 counteracts Budd Chiari syndrome in rats.

Key words: BPC 157; Budd Chiari syndrome; Portal/caval hypertension

Core tip: To demonstrate that pentadecapeptide BPC 157 can resolve Budd Chiari syndrome in rats, we provided gross, microscopy, nitric oxide-, malondialdehyde-liver levels, serum enzymes, electrocardiogram, portal, caval hypertension, aortal hypotension, thrombosis, hepatomegaly, splenomegaly and venography assessments. BPC 157 counteracts increased P wave amplitude, tachycardia and ST-elevation (*i.e.*, right heart failure; acute thrombotic coronary occlusion). Bypassing pathway (inferior caval vein-azygos (hemiazygos) vein-superior caval vein and portocaval shunt) rapidly appears. Even with the severe caval > portal hypertension, BPC 157 counteracts portal and caval hypertension and aortal hypotension. BPC 157 counteracts refractory ascites. Portal vein tributaries, inferior caval vein, hepatic and coronary arteries thrombosis was counteracted. In addition, there are counteracted severe lung pathology, liver, intestine hemorrhagic lesion, increased liver and spleen weight. During ligation-period, nitric oxide- and malondialdehyde-level in liver remained within normal healthy values, and increased serum enzymes markedly lessened. In conclusion, BPC 157 counteracts Budd Chiari syndrome in rats.

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INTRODUCTION

Here we raised new hypothesis about rapid bypassing of the suprahepatic inferior caval vein occlusion in the Budd-Chiari syndrome (BCS) in rats along with the active and effective pharmacotherapy treatment.

In this, we perceive BCS as originally suggested[1,2], a hepatic venous outflow obstruction and its manifestation, regardless of cause, but mostly attributable to thrombosis, which can be located anywhere from the small hepatic veins until the

entrance of the inferior caval vena into the right atrium[1,2]. Thereby, with some limitations[3], since in the rat it is almost impossible to dissect the hepatic veins[3], few rats studies used occluding the inferior vena cava cranially to the hepatic veins[2,4-7], but bypassing of the occlusion in the rat BCS along with pharmacotherapy treatment was not considered.

Recently, as a possible therapy resolving solution, pentadecapeptide BPC 157 therapy[8-20], has been used in alleviating vascular occlusion disturbances[21-25]. BPC 157 was previously reviewed as novel mediator of Robert cytoprotection and endothelium protection in the stomach[8-11], and gut-brain axis[12], beneficial therapy in gastrointestinal tract[13,14], with particular reference to vascular recruitment[11,15-17], ulcerative colitis[14] and tumor cachexia[18], and other tissues healing[19,20].

Rapid activation of a bypassing loop[21-25] occurs in rats with an infrarenal ligation of the inferior vena cava, relieving a Virchow's triad situation[21], much like in rats with ischemic/reperfusion colitis[22], duodenal venous congestion lesions[23], perforated cecum[24], bile duct ligation induced liver cirrhosis and portal hypertension[25].

Specifically, there is prevention and reversal of both caval and portal hypertension and aortal hypotension, tachycardia, thrombosis and thrombocytopenia, and consequently reduced prolonged bleeding. In addition, there is both preserved and rescued intestinal mucosal integrity and vein integrity, reduced malondialdehyde (MDA), even to normal levels in both ischemic and reperfusion conditions in tissues (*i.e.*, liver, colon, duodenum, cecum and veins) and plasma[21-25].

In this current study, we assessed whether BPC 157 therapy compensated for the complete suprahepatic occlusion of the inferior vena cava, an immediate obstructive inferior vena cava and portal syndrome. In these obstructions, it would activate an azygos/hemiazygos vein bypassing pathway, and upgrade an inadequate rescuing inferior-superior vena cava shunt to an adequate one, as well as a portocaval shunt. With BCS in rats, both caval and portal hypertension, and aortal hypertension occurred. BPC 157 therapy was assessed in counteracting rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST-elevation, tachycardia, gross organ lesions, and liver and spleen weight increases.

Notably, BPC 157 was originally used as an anti-ulcer agent, being stable and maintaining a native configuration in human gastric juices for more than 24 h, as previously reviewed[8-20]. It has been used in trials for ulcerative colitis and multiple sclerosis, with a very safe profile (LD1 not achieved), as previously reviewed[8-20]. Furthermore, as an important conceptual and practical activity point, in previous studies[8-20], BPC 157 was thought to be a novel mediator of Robert's cytoprotection, the concept of a compound providing epithelium and endothelium protection, maintaining gastrointestinal mucosal integrity and organoprotection, as previously reviewed[8-20]. Likely, these beneficial effects might additionally contribute to the suggested therapeutic effect related to vessel recruitment in bypassing occlusions. As such, we investigated pentadecapeptide BPC 157 in resolving short and long-lasting BCS in rats.

MATERIALS AND METHODS

Animals

This study was conducted with 12-wk-old, 200 g birth weight , male albino Wistar rats, randomly assigned at 6 rats/group/interval. Rats were bred in-house at the Pharmacology animal facility, School of Medicine, Zagreb, Croatia. The animal facility was registered by the Directorate of Veterinary (Reg. No: HR-POK-007). Laboratory rats were acclimated for five days and randomly assigned to their respective treatment groups. Laboratory animals were housed in polycarbonate (PC) cages under conventional laboratory conditions at 20–24 °C, relative humidity of 40%–70% and noise level 60 dB. Each cage was identified with dates, number of study, group, dose, number and sex of each animal. Fluorescent lighting provided illumination 12 hours per day. Standard good laboratory practice (GLP) diet and fresh water was provided ad libitum. Animal care was in compliance with standard operating procedures (SOPs) of the Pharmacology animal facility, and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

This study was approved by the local Ethic Committee. Ethical principles of the study complied with the European Directive 010/63/E, the Law on Amendments to the Animal Protection Act (Official Gazette 37/13), the Animal Protection Act (Official Gazette 135/06), the Ordinance on the protection of animals used for scientific purposes (Official Gazette 55/13), Federation of European Laboratory Animal Science

Associations recommendations and the recommendations of the Ethics Committee of the School of Medicine, University of Zagreb. The experiments were assessed by observers blinded as to the treatment.

Drugs

Medication was administered as described previously[21-25], without use of a carrier or peptidase inhibitor, for stable gastric pentadecapeptide BPC 157, a partial sequence of the human gastric juice protein BPC, which was freely soluble in water at pH 7.0 and in saline. BPC 157 (GEPPPGKPADDAGLV, molecular weight 1419; Diagen, Slovenia) was prepared as a peptide with 99% high-performance liquid chromatography purity, with 1-des-Gly peptide being the main impurity. The dose and application regimens were as described previously[8-20].

Surgery

Rats were deeply anesthetized with intraperitoneal (ip) injected 40 mg/kg thiopental (Rotexmedica, Germany) and 10 mg/kg diazepam (Apaurin; Krka, Slovenia) The suprahepatic inferior vena cava was then exposed *via* a midline laparotomy, and was occluded by ligation. Rats were maintained for the next 15 min, 24 h or 48 h.

Medication

In rats with suprahepatic occlusion of the inferior vena cava, in evaluating lesions and blood vessels by gross and microscopic assessment, electrocardiogram (ECG), thrombosis, serum enzyme levels and oxidative stress (MDA and nitric oxide, NO levels) in liver tissue, rats were treated with 10 μg/kg BPC 157, 10 ng/kg BPC 157, or 5mL/kg saline as an abdominal bath at 1 min ligation-time. In addition, for portal vein, vena cava, and abdominal aorta pressure recordings 10 μg/kg BPC 157, 10 ng/kg BPC 157 or 5 mL/kg saline was applied in rats, either intragastrically or as an abdominal bath, at 15 minutes, 24 h or 48 h ligation-time. For venography, 10 μg/kg BPC 157, 10 ng/kg BPC 157 or 5 mL/kg saline was applied as an abdominal bath, at 15 minutes ligation-time, just before venography.

Portal and caval vein and abdominal aorta pressure recording

Recordings were made in deeply anesthetized and laparatomized rats, with a cannula (BD Neoflon™ Cannula) connected to a pressure transducer (78534C MONITOR/TERMINAL; Hewlett Packard, United States) inserted into the portal vein, inferior vena cava and abdominal aorta at the level of the bifurcation at 15 min, 24 h or 48 h post-ligation. Each recording lasted five minutes, being assessed in one minute intervals.

Notably, normal rats exhibited a portal pressure of 3–5 mmHg[25] similar to that of the inferior vena cava, though with at least 1 mmHg higher values in the portal vein. By contrast, abdominal aorta blood pressure values were 100–120 mmHg at the level of the bifurcation[21].

ECG recording

ECGs were recorded continuously in deeply anesthetized rats for all three main leads, by positioning stainless steel electrodes on all four limbs using an ECG monitor with a 2090 programmer (Medtronic, United States) connected to a Waverunner LT342 digital oscilloscope (LeCroy, United States). This arrangement enabled precise recordings, measurements and analysis of ECG parameters[21].

Vessels, intestine, liver, spleen, ascites presentation

The presentation of the vessels was recorded in deeply anaesthetized rats, with a camera attached to a VMS-004 Discovery Deluxe USB microscope (Veho, United States). We assessed vessels, as to whether they had afilled or cleared out (hollow) appearance at the stomach, and for arcade vessels on the ventral and dorsal sides in 1 cm long segments of duodenum, jejunum, ascending colon, and for 10 vessels from the proximal to distal cecum throughout the experiment. The assessments occurred at selected time points before and after therapy, in rats with a suprahepatic occlusion of the inferior vena cava, at 5, 10 and 15 min post-ligation.

At 15 min, 24 h and 48 h post-ligation, we assessed hemorrhagic congestive areas in the stomach, duodenum, jejunum, cecum and ascending colon. Scoring was based on opening (1, normal mucosa presentation; 2, only small hemorrhagic areas; 3, advanced hemorrhagic areas; and 4, extensive and severe hemorrhagic areas) and azygos vein presentation (1, moderate decrease; 2, mild decrease; 3, not different from healthy; and 4, abundant increase). Liver and spleen weights were expressed as a percent of the total body weight (for normal rats, liver 3.2%–4.0% and spleen 0.20%–0.26%). Ascites (mL) was also assessed.

Venography

Venography was performed in rats with a suprahepatic occlusion of the inferior vena cava at 15 min post-ligation, using a C-VISION PLUS fluoroscopy unit (Shimadzu, Japan)[18]. Two ml (0.3 mL/s) warmed Omnipaque 350 (iohexol) non-ionic contrast medium (GE Healthcare, United States) was injected into the inferior vena cava at the level of bifurcation of rats with a suprahepatic occlusion of the inferior vena cava. The contrast medium was visualized under real-time to ensure adequate filling. A subtraction mode was used to record the images at 14 frames per second. At 15 minutes post-ligation, venograms were taken, captured, and digitized into files on a personal computer and were analyzed using ISSA image software (Vamstec, Croatia). Rats having a full presentation of the azygos vein and portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior vena cava pathway were assessed.

Microscopy

Tissue specimens from liver, spleen, stomach, duodenum, ileum, cecum, ascending colon, cecum, lungs and heart were obtained from rats with suprahepatic occlusion of the inferior vena cava at 15 min, 24 h and 48 h post-ligation. These were fixed in buffered formalin (pH 7.4), for 24 h, dehydrated, and embedded in paraffin wax. The samples were stained with hematoxylin-eosin. Tissue injury was evaluated microscopically by a blinded examiner.

Thrombus assessment

On being euthanized, the portal vein, superior mesenteric vein (up to the inferior anterior pancreaticoduodenal vein), splenic vein and inferior vena cava, as well as the hepatic and coronary arteries were removed from the rats, and clots were weighed[21].

Bilirubin and enzyme activity

To determine the serum levels of aspartate transaminase, alanine transaminase (IU/L), and total bilirubin (μmol/L), blood samples were collected immediately after euthanasia and were centrifuged for 15 min at 3000 rpm. All tests were performed using an Olympus AU2700 analyzer with original test reagents (Olympus Diagnostics, Ireland)[26-32]. However, since there was no increase in bilirubin, the data were not shown.

Oxidative stress in liver

Oxidative stress was assessed in collected tissue samples at 15 min, 24 h and 48 h post-ligation, by quantifying thiobarbituric acid-reactive species (TBARS) as MDA equivalents, as previously described[21-25]. The tissue samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) containing 0.1 mmol/L butylated hydroxytoluene (BHT, TissueRuptor; Qiagen, United States) and sonicated for 30 s in an ultrasonic ice bath (Branson, United States). Trichloroacetic acid (TCA, 10%) was added to the homogenate, the mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was collected. Thiobarbituric acid (TBA, 1%) was added, and the samples were heated at 95 °C for 60 min. The tubes were then kept on ice for 10 min. Following centrifugation (14000 rpm, 10 min), the absorbance of the mixture was determined at a wavelength of 532 nm. The concentration of MDA was read from a standard calibration curve, plotted using 1,1,3,3'-tetraethoxy propane. The extent of lipid peroxidation was expressed as MDA equivalents, using a molar extinction coefficient for MDA of 1.56 × 105 mol/L/cm. Protein concentration was determined using a DC Protein Assay Kit, (Bio-Rad, United States). Results were expressed in nmol per mg protein.

NO determination in liver

NO- levels in liver tissue samples were determined at 15 min, 24 h and 48 h post-ligation, using the Griess reaction (Griess Reagent System; Promega, United States). Sulfanilamide was incubated with the homogenized tissue, and then, N-1-naphthyl ethylenediamine dihydrochloride was added. The Griess reaction was based on a diazotization reaction in which acidified nitrite reacted with diazonium ions and, in a further step, was coupled to N-1-naphthyl ethylenediamine dihydrochloride, forming a chromophoric azo derivate. Absorbance was measured at 540 nm, using sodium nitrite solution as a standard. NO-levels were reported in μmol/mg protein. The protein concentrations were determined using a DC Protein Assay Kit, (Bio-Rad, United States), as described previously[21-25].

Statistical analysis

Statistical analysis was performed by parametric one-way analysis of variance, with post-hoc Newman-Keuls test and non-parametric Kruskal-Wallis test and

subsequently the Mann-Whitney *U* test to compare groups. Values were presented as the mean ± SD and as the minimum/median/maximum. To compare the frequency difference between groups, the χ^2 test or Fischer's exact test was used. $P < 0.05$ was considered statistically significant.

RESULTS

All rats with suprahepatic occlusion of the inferior vena cava in BPC 157-treated groups exhibited a full presentation of the azygos vein and portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior vena cava pathway, unlike the controls, (Fisher's exact probability test $P < 0.05$ compared to control; Figure 1). This justified the focus of this current study on the stable gastric pentadecapeptide BPC 157 and rapid recovery of all hemodynamic disturbances in Budd-Chiari-rats, the severe portal and caval hypertension and abdominal aorta hypotension and full course of BCS model.

All BPC 157 administration regimens (10 μg/kg and 10 ng/kg, abdominal bath, and intragastric applications) were effective in rats with BCS (Figures 1-11). Indicative of how BPC 157 might affect the course of BCS (Figure 1) and reversed the course of disturbances, marked attenuation occurred when it was given at 15 min post-ligation, much as in rats with prolonged BCS (at 24 h and 48 h post-ligation). Therefore, preexisting severe portal and caval hypertension and systemic hypotension (seen in the abdominal aorta), either short-lasting or long-lasting, rapidly responded to any of the BPC 157 regimens (Figure 2). In addition, over prolonged periods, the worsening that simultaneously appeared and persisted, the high portal hypertension, and particularly, the caval hypertension and aortic hypotension were not compensated; however, these completely disappeared with BPC 157 medication (Figure 2). Similarly, controls presented with immediately peaked *P* values, significant ST-elevation and tachycardia, as identifiers of acute thrombotic coronary occlusion and right heart failure (Figure 3), which rapidly disappeared under all the BPC 157 regimens (Figure 3). As visualized grossly in the early and prolonged periods, with BPC 157 treatment, increased blood vessel branching rapidly appeared in the serosa of all organs affected (Figures 6 and 8), with hemorrhagic lesions being markedly attenuated in the stomach, duodenum, jejunum, ileum, cecum and ascending colon (Figures 5 and 7), as well as reducing hepatomegaly and splenomegaly (Figure 5). There was a particular presentation of the azygos vein as an indication of the counteraction of right heart malfunction and the reestablishing of blood flow continuity between caval veins (Figure 8). Indicative of a reversal of BCS, while there was progressive thrombosis in controls in the portal vein, splenic vein, superior mesenteric vein, and inferior vena cava, as well as in the hepatic and coronary arteries, strong attenuation occurred in the veins and arteries of BPC 157-treated rats, where considerably smaller clots were observed (Figure 4). In addition, BPC 157 rats had much less ascites (Figure 5), and while serum alanine transaminase and aspartate transaminase values increased in controls, they were lower in rats treated with BPC 157 at all time intervals (Figure 5).

Increased MDA- and NO-levels in the liver may be indicative of BCS. BPC 157 administration resulted in MDA- and NO-levels in the liver, close to or within a normal healthy range in both early and prolonged periods of ligation (Figure 6).

Rats with BCS regularly showed considerable lesions early post-ligation. For example, in liver, there was substantial congestion of the central vein, branches of the terminal portal venules, and sinusoidal dilatation in the controls, while there was far less congestion in BPC 157-treated rats (Figure 9). In the spleen, there was sinusoidal congestion, and dilatation and enlargement of red pulp leading to reduction of white pulp, features, which were less apparent in BPC 157-treated rats (Figure 9). In stomach of controls, there was substantial congestion and dilatation of mucosal and submucosal capillaries, submucosal edema, ischemic changes, such as architectural distortion and foci of hemorrhage with fibrin deposition. Duodenal lesions in controls were characterized by substantial capillary congestion with prominent ischemic changes, architecture distortion and loss of crypts, with foci of hemorrhage, edema of the lamina propria and mild lymphocytic infiltration and ischemic changes, such as architectural distortion and foci of hemorrhage with fibrin deposition (Figure 10). By contrast, only very mild capillary congestion was found in the stomach and duodenum of BPC 157-treated rats, with some reparatory changes to epithelium. In the jejunum, ileum, cecum and colon of control rats, substantial capillary congestion with mild ischemic changes, loss of crypts with foci of hemorrhage, edema of the lamina propria and mild lymphocytic infiltration were present. By contrast, these effects were much less expressed in BPC 157-treated rats; in particular, there was

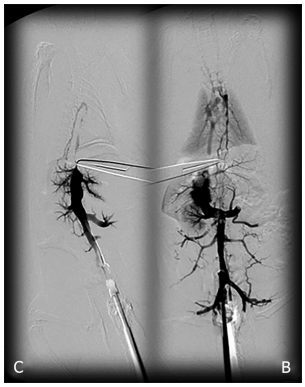

Figure 1 BPC 157 and venography assessment in rats with a suprahepatic occlusion of the inferior vena cava. Inferior vena cava venography at the level of the bifurcation at 15 minutes ligation time. Blood flow through the inferior vena cava through the azygos vein to the superior vena cava, portal vein to superior-inferior mesenteric vein-rectal vein-left iliac vein-inferior vena cava in BPC 157-treated rats (B), but not in the controls (C).

preservation of architecture and scattered lymphocytes with little to no ischemic changes, and some reparatory changes of epithelium (Figure 11). Finally, in the lungs, control rats showed edema of the interstitium, substantial dilatation and congestion of capillaries in the alveolar septum, with few to many lymphocytes, while in BPC 157-treated animals, we found little to no edema of the interstitium, very few lymphocytes, and only mild capillary congestion (Figure 9). In addition, as expected, no morphological changes were found in the myocardium due to the fact that changes found on ECGs were the result of acute right ventricular overload (data not shown).

DISCUSSION

We adopted that regardless of cause, Budd–Chiari syndrome hepatic venous outflow obstruction can be located anywhere from the small hepatic veins until the entrance of the inferior caval vena into the right atrium[1,2], as originally suggested[1,2]. Thereby, with the suprahepatic occlusion of the inferior caval vein, which was complete (and thereby, abrupt initiation), and its manifestations, as a model of the BCS in rats[2-7], and recovery in the BPC 157-treated rats, we extended evidence from previous vascular occlusion studies[21-25]. As a new point, BPC 157 therapy rapidly activated bypassing pathways a caval shunt (inferior vena cava-azygos vein-superior vena cava shunt) or a portocaval shunt (portal vein-superior-inferior mesenteric vein-rectal vein-inferior vena cava). Severe post-hepatic portal hypertension and caval hypertension was eliminated (and aortal hypotension as well), by BPC 157 given at 15 min, 24 h or 48 h, appearing to be successful as a deep vein thrombosis and ischemic/reperfusion therapy[21-25].

By contrast, in the control Budd-Chiari-rats, the failed vena cava shunt (*i.e.*, azygos vein) ineffectively competes against resolving the condition of rats, with severe portal hypertension (> 60 mmHg) and caval hypertension (> 70 mmHg in the later period). A gradient of at least 15 mmHg as portal hypertension reflects a decrease in regular venous return to the heart and failure of spontaneous decompression of the portal system. These disturbances might be more than in humans, with the persistence of the left superior vena cava, the azygos vein on the left of the aorta and vertebral column, and the hemiazygos vein likely lacking[33]. For example, there is the higher range of this portal hypertension versus the long-lasting portal hypertension in rats drinking alcohol (> 25 mmHg)[26] or from bile duct ligation-induced liver cirrhosis (> 18 mmHg)[25]. Likewise, there is a higher range of caval hypertension versus inferior vena cava infrarenal occlusion-induced caval hypertension (> 25 mmHg)[21]. Severe aortal hypotension was not compensated, but progressed, unless BPC 157 was given.

Thus, the therapy effect overrode the high resistance to the therapy, which had to be more than the portal vein-stenosis with the slow progression of mild portal hypertension in rats (< 20 mmHg)[34-36]. Therefore, counteraction of the described abrupt BCS necessitated the pleiotropic beneficial effects of treatments such as BPC 157 that might be advantageous to counteract the whole syndrome, as previously reviewed[8-20].

Thereby, there were in particular beneficial effects on the liver, including on portal hypertension, and on the intestinal tract[26-32], lung[37-39], thrombosis[21,40], venous[21] and arterial[40], and heart disturbances[41-45], free radicals formation[46-49] and free radical-

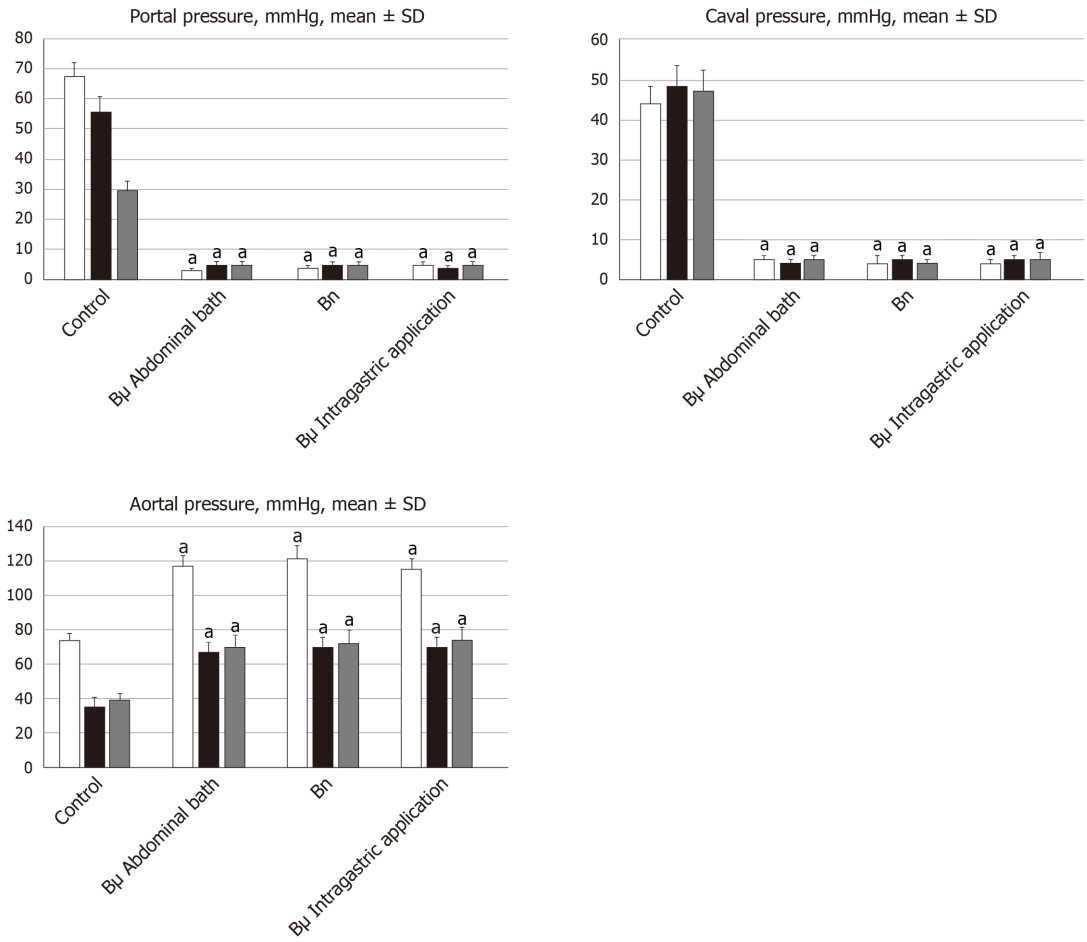

Figure 2 Antagonizing effect of BPC 157 on portal and caval hypertension, and aortal hypotension. Portal, caval and aortal pressure (mmHg), mean ± SD. For portal vein, vena cava, and abdominal aorta pressure recordings, medication of BPC 157 [10 μg/kg (Bμg), 10 ng/kg (Bng)] or saline (5 mL/kg; control) was applied in rats intragastrically or as an abdominal bath, at 15 min (white bars), 24 h (black bars) or 48 h (gray bars) post-ligation. $^{a}P < 0.05$ *vs* control.

induced lesions[46-50]. These appear in addition to the described effect on vessel recruitment[21-25], with the azygos vein readily presented in BPC 157-treated rats compared to empty and tortuous examples in the controls (Figure 8).

For BPC 157-treated rats, in early and in late periods, the level of oxidative stress in the liver (MDA- and NO-levels, otherwise known to be increased and critical mediators of liver fibrosis[25]) was almost continuously within normal values as it was in rats with bile duct occlusion[25]. By contrast, along with previous studies[21-25,46-49], in control livers, high MDA- and NO-values during suprahepatic occlusion of the inferior vena cava persisted (Figure 6). Control rats exhibited rapid clot formation, generalized thrombosis (in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery and cardiac artery; Figure 4), and ascites (consistently present in the early and late period; Figure 5). BPC 157-treated rats exhibited counteraction of these effects, with less venous and arterial clots (as stasis is eliminated or largely attenuated) and reduced ascites, as had previously been emphasized[21-25,40].

In addition, controls presented with immediately peaked *P* values, significant ST-elevation and tachycardia, as identifiers of acute thrombotic coronary occlusion and right heart failure (Figure 3), and thereby, lung congestion (Figure 11). BPC 157-treated rats had the ECG disturbances completely abrogated, and less lung congestion. Therefore, the immediate presentation of adverse effects and the parallel therapeutic effects might suggest an essential cause-consequence chain of events of Budd-Chiari and BPC 157 in rats, and in particular, a relation to the liver as the prime organ affected[26-32] and failure of its circulation. All controls exhibited gross hemorrhagic lesion progression, hepatomegaly and splenomegaly (Figures 5 and 7), with gross bleeding in the stomach and duodenum, but also intestinal perforation, increased serum enzyme values (Figure 5), and a dilated central vein and terminal portal venules (Figure 9). This contrasted with the spared gross liver presentation, counteracted hepatomegaly and splenomegaly, reduced gross lesions in the stomach,

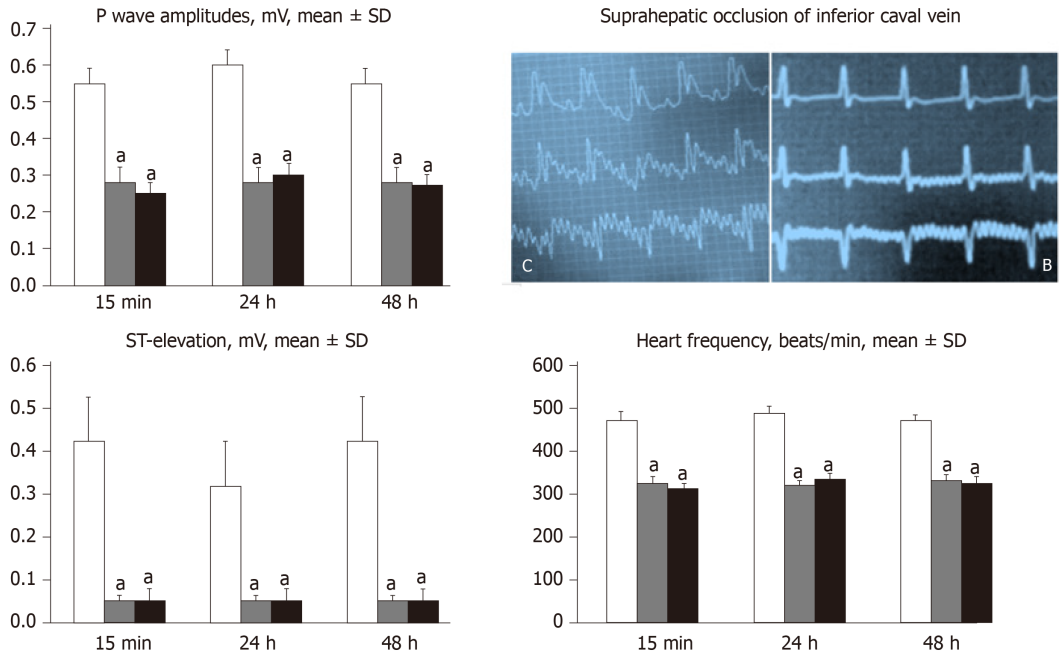

Figure 3 Counteracting effect of BPC 157 in rats with a suprahepatic occlusion of the inferior vena cava on peaked P waves, sinus tachycardia, ST-elevation 15 min post-ligation [controls (*C*), BPC 157 rats (*B*)] and assessment at 15 min, 24 h and 48 h ligation-time, mean ± SD. Treatment with 10 μg/kg BPC 157 (gray bars), 10 ng/kg BPC 157 (black bars), or saline (1 mL/rat; white bars) as a bath administration given at 1 min post-ligation. [a]*P* < 0.05 *vs* control.

duodenum and intestine, lower serum enzyme values, and no congestion of the central vein or branches of the terminal portal venules in BPC 157-treated rats. In particular, hepatic artery patency might be essential, and indicative for the thrombosis counteraction in all of the investigated veins and arteries, which were investigated[21,40]. Note, hepatic artery perfusion could be essential for recovery from hepatic venous outflow obstruction in rats, pointed out when usually occurring after 70% hepatectomy and right median hepatic vein ligation, in which the role of hepatic artery inflow is of particular importance, in the condition likely even more severe than the obstruction of the hepatic veins by ligation of the upper part of inferior caval vein[51].

Besides, BPC 157 therapy effects described in rats with bile duct cirrhosis and portal hypertension resulted in much less Ki-67- and alpha smooth muscle actin (α-SMA) staining, counteracted disturbed cell proliferation and cytoskeletal structure in hepatic stellate cells, and showed a less disturbed collagen presentation, and thereby, less Mallory staining, and more hepatocytes where fewer had double nuclei[25]. In the course of this cholestatic model of liver injury[25], BPC 157 also counteracted all characteristic structural, biochemical, and hemodynamic disturbances, with portal hypertension either not developed, portal pressure remaining normal (BPC 157 prophylactic regimen) or portal hypertension rapidly reversed (delayed post-treatment). In addition, further consideration might be given to the counteraction of increased liver values of interleukin-6, tumor necrosis factor alpha and interleukin-1β[25], and the relation with counteracted tumor cachexia[18], muscle wasting, increased pro-cachectic and pro-inflammatory mediators, and changes in the expression of forkhead box O3, phosphorylated protein kinase B, phosphorylated mammalian target of rapamycin, and phosphorylated glycogen synthase kinase 3β[18]. This indicates the interaction of BPC 157 with several molecular pathways[52-58], in particular with increased expression and internalization of vascular endothelial growth factor receptor 2 and the activation of the vascular endothelial growth factor receptor 2-Akt- endothelial NO synthase signaling pathway, without the need for other ligands or shear stress[54].

Finally, there is a "honeycomb" smaller vessel network, which appears at the intestinal serosa, which was also noticed in rats with ischemic/reperfusion colitis[23], duodenal venous congestion lesions[24], or inferior vena cava occlusion[21]. Thus, the counteraction of hemorrhagic intestinal lesions, from the stomach to the ascending colon, occurs as a very rapid activation of a bypassing loop, thereby reducing portal hypertension. Otherwise, intestinal ischemia[59] appears as the final consequence of blood pooling in the splanchnic bed, inducing portal hypertension, and multivisceral edema, and mesenterial venous occlusion, could also induce intestinal injury as early as within 5 min. Both of these effects are inflow and outflow alterations[60].

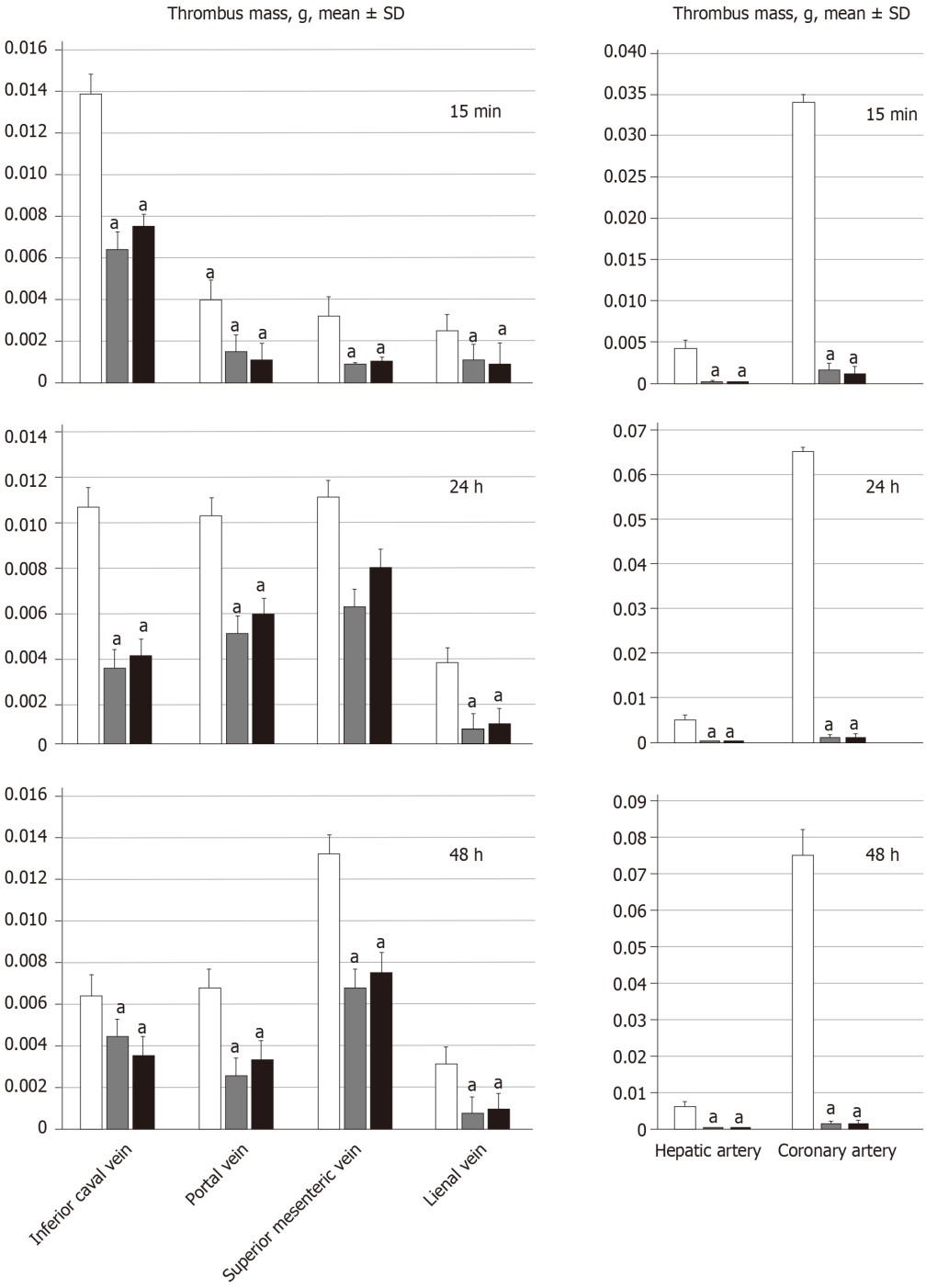

Figure 4 BPC 157 attenuates thrombus presentation (thrombus mass, g) in veins (inferior vena cava, portal vein, superior mesenteric vein, and splenic vein, SV) and arteries (hepatic artery and coronary artery) following suprahepatic occlusion of the inferior vena cava. Assessment at 15 min, 24 h and 48 h post-ligation; mean ± SD. Treatment with 10 μg/kg BPC 157 (gray bars), 10 ng/kg BPC 157 (black bars), or saline (1 mL/rat; white bars) as a bath administration given at 1 min post-ligation. $^{a}P < 0.05$ *vs* control.

Concluding, BPC 157 therapy (intragastric or abdominal bath, 10 μg/kg and 10 ng/kg dosages), using a previously described protocol[21-25], could alleviate suprahepatic occlusion of the inferior caval vein, as model of the BCS in rats, activating bypassing pathways. BPC 157 likely counteracted Virchow's triad, as it did in rats with infrarenal inferior vena cava occlusion[21]. A particular effect on portal hypertension disturbances, by activation of bypassing pathways, might be envisaged that needs further elaboration. For example, BPC 157 might specifically interact with the NO-system, as previously reviewed[11,17]. In rats that received NO-agents, L-arginine methyl ester (L-NAME; a NOS blocker) or L-arginine (NOS-substrate), BPC 157 counteracted L-NAME-induced hypertension, as well as L-arginine-induced hypotension[61]. This effect appeared to counteract potassium overdose, severe

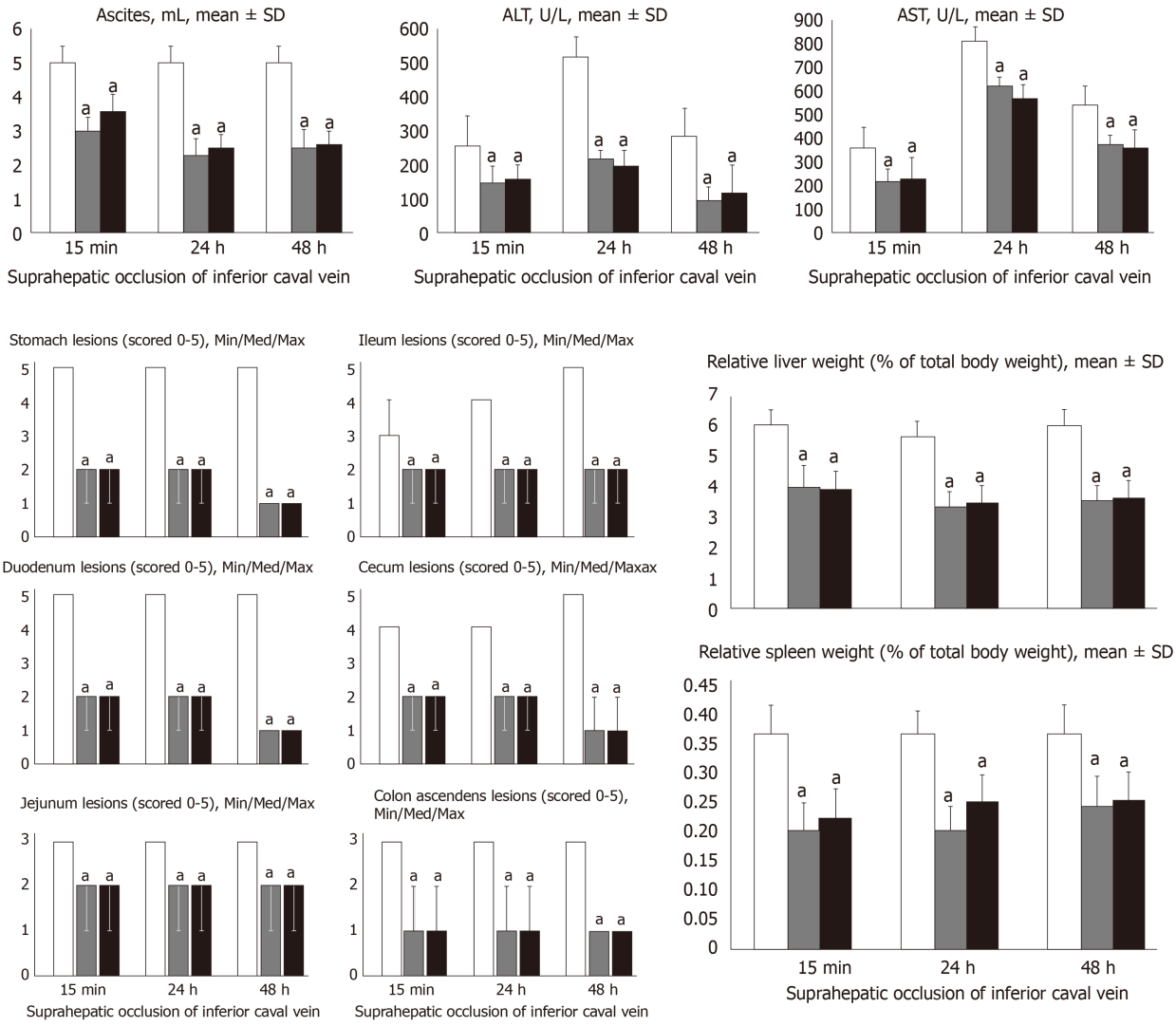

Figure 5 BPC 157 counteracts ascites (mL), serum enzyme levels (U/L), hepatomegaly and splenomegaly (mean ± SD), stomach, duodenal, jejunal, ileal, cecal and ascending colon lesions (scored 0–5; Min/Med/Max), following suprahepatic occlusion of the inferior vena cava. Assessment at 15 min, 24 h and 48 h post-ligation. Treatment with 10 μg/kg BPC 157 (gray bars), 10 ng/kg BPC 157 (black bars), or saline (1 mL/rat; white bars) as a bath administration given at 1 min ligation. [a]*P* < 0.05 *vs* control.

hyperkalemia arrhythmias and hypertension[41] or doxorubicin induced chronic heart failure and hypotension[43]. Finally, as a novel understanding of the cytoprotective mechanisms that are essential for its activity, as previously reviewed[8-20], BPC 157 has recently been shown to increase the survival of cultured enteric neurons and the proliferation of cultured enteric glial cells; therefore healing of a damaged enteric nervous system might also contribute to the observed therapeutic effects[62]. These effects may together counteract a downward spiral in rats with BCS, in acute states, much as in prolonged ischemia.

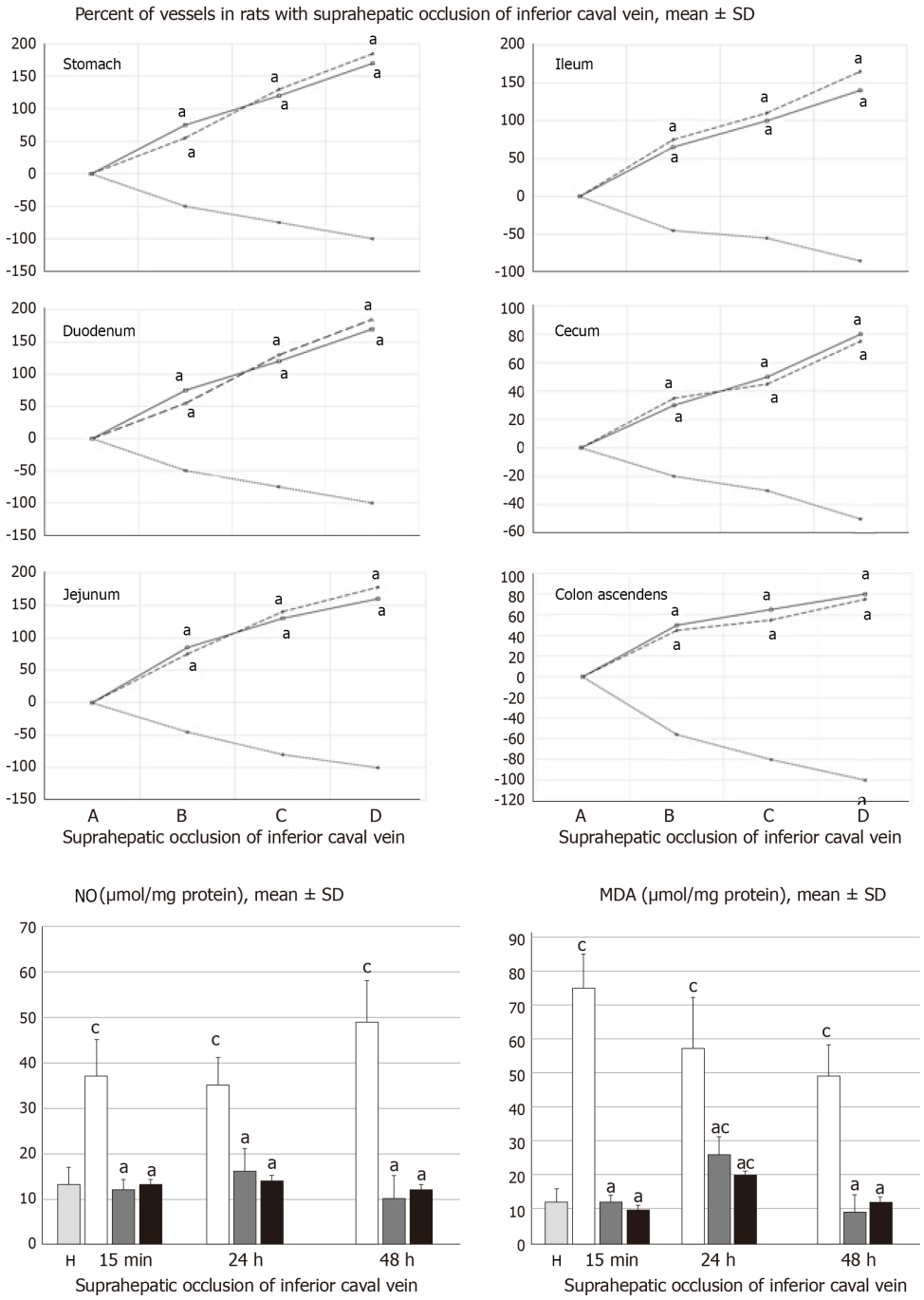

Figure 6 BPC 157 improves vessel presentation following suprahepatic occlusion of the inferior vena cava (upper), and eliminates or attenuates increased nitric oxide and malondialdehyde levels (nmol/mg protein) in liver. Vessels were assessed as to whether they were (filled or cleared out at the stomach, and between the arcade vessels on the ventral side for 1 cm long segments of duodenum, jejunum and ascending colon, and between 10 vessels from the proximal to distal cecum. Assessment was made throughout the experiment, with the point (A) immediately before therapy considered to be 100%, at selected time points before (A) and after therapy (B, C and D) in rats with suprahepatic occlusion of the inferior vena cava at 5 (B), 10 (C) and 15 (D) minutes post-ligation (mean ± SD). The gross appearance of the tissue was recorded using a USB microscope camera (upper). Counteraction of the increased nitric oxide and malondialdehyde levels in liver (lower) at 15 min, 24 h and 48 h post-ligation. Treatment with 10 μg/kg BPC 157 (gary bars), 10 ng/kg BPC 157 (black bars), or saline (1 mL/rat; white bars) as a bath administration given at 1 min post-ligation. $^{a}P < 0.05$ *vs* saline; $^{c}P < 0.05$ *vs* healthy liver (H). NO: Nitric oxide; MDA: Malondialdehyde.

Figure 7 Gross presentation of lesions (*C*) and lesions attenuation (*B*) in rats with suprahepatic occlusion of inferior vena cava. Lesions were assessed using a camera attached to a USB microscope (Veho Discovery Deluxe VMS-004), at 15 min (upper, *a*), 24 h (middle, *b*), and 48 h (low, *c*) post-ligation in the gastrointestinal tract. From left to right, the stomach, duodenum, jejunum, ileum, cecum and ascending colon on treatment with saline (*C*) or BPC 157 (*B*).

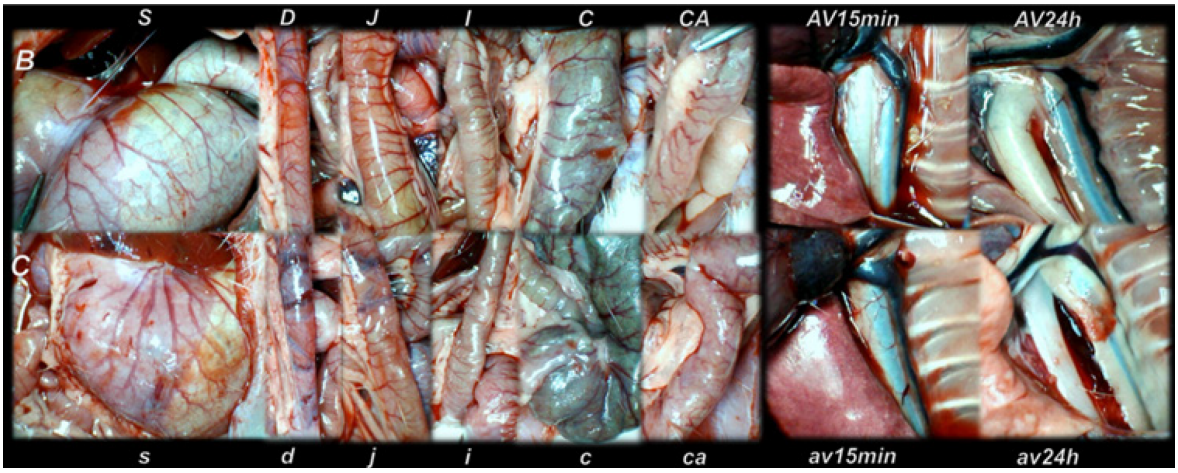

Figure 8 Gross vessel presentation in rats treated with saline (*C*, small letters, lower) and improvement in rats treated with BPC 157 (*B*, capitals, upper). Assessments were made using a camera attached to a USB microscope (Veho Discovery Deluxe VMS-004) in the stomach (*s*/*S*), duodenum (*d*/*D*), jejunum (*j*/*J*), ileum (*i*/*I*), cecum (*c*/*C*) and ascending colon (*ca*/*CA*) of rats with suprahepatic occlusion of the inferior vena cava at 15 min post-ligation (left), and azygos vein presentation at 15 min (*av15min*/*AV15min*) and 24 h (*av24h*/*AV24h*) post-ligation (right).

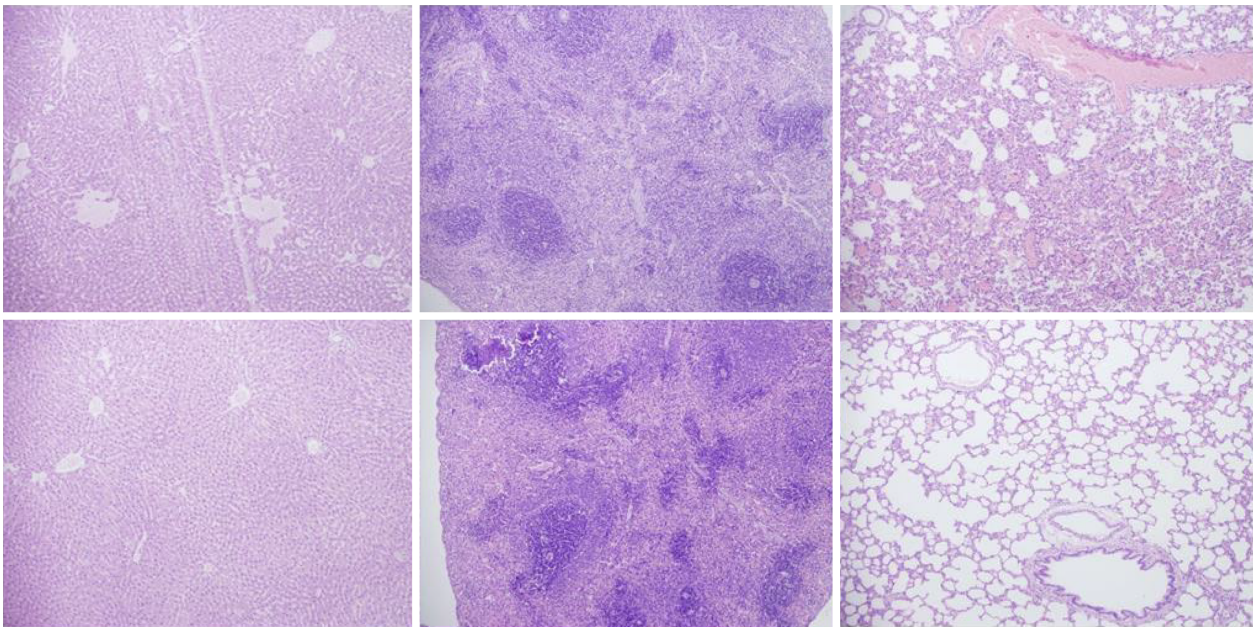

Figure 9 Histological presentation in rats with suprahepatic ligation of the inferior vena cava. Assessment (stained with hematoxylin-eosin, magnification x 10, scare bar: 50 μm) was made at 48 h post-ligation, in controls (upper) and BPC 157-treated rats (lower), liver (left upper, control; lower, BPC 157), spleen (middle upper, control; lower, BPC 157), and lungs (right upper, control; lower, BPC 157). Note, the substantial congestion of the central vein, branches of the terminal portal venules, and sinusoidal dilatation in liver, sinusoidal congestion, dilatation and enlargement of red pulp leading to reduction of white pulp in spleen, edema of the interstitium, substantial dilatation and congestion of capillaries in the alveolar septum in lungs (upper), which were markedly counteracted in BPC 157-treated rats (lower).

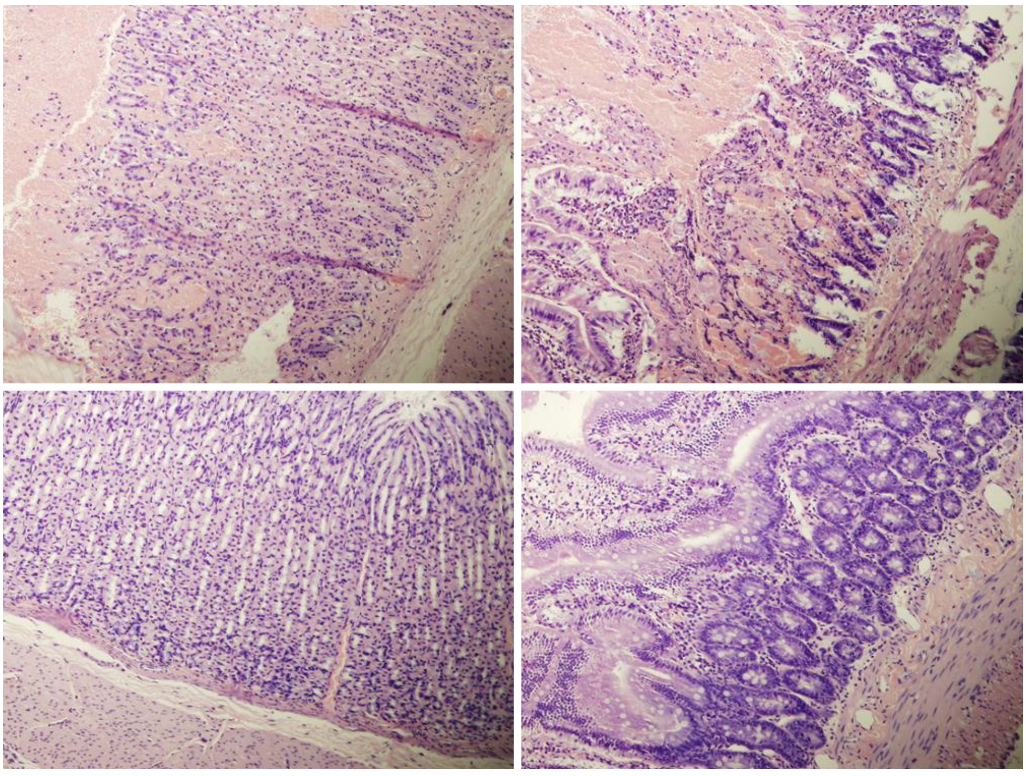

Figure 10 Histological presentation in rats with suprahepatic ligation of the inferior vena cava. Assessment (stained with hematoxylin-eosin, magnification x 10, scare bar: 50 μm) was at 48 h post-ligation, in controls (upper) and BPC 157-treated rats (lower), stomach (left upper, control; lower, BPC 157), duodenum (right upper, control; lower, BPC 157). Note, substantial congestion and dilatation of mucosal and submucosal capillaries, submucosal edema, ischemic changes, such as architectural distortion and foci of hemorrhage with fibrin deposition in controls (upper), which were markedly counteracted in BPC 157-treated rats (lower).

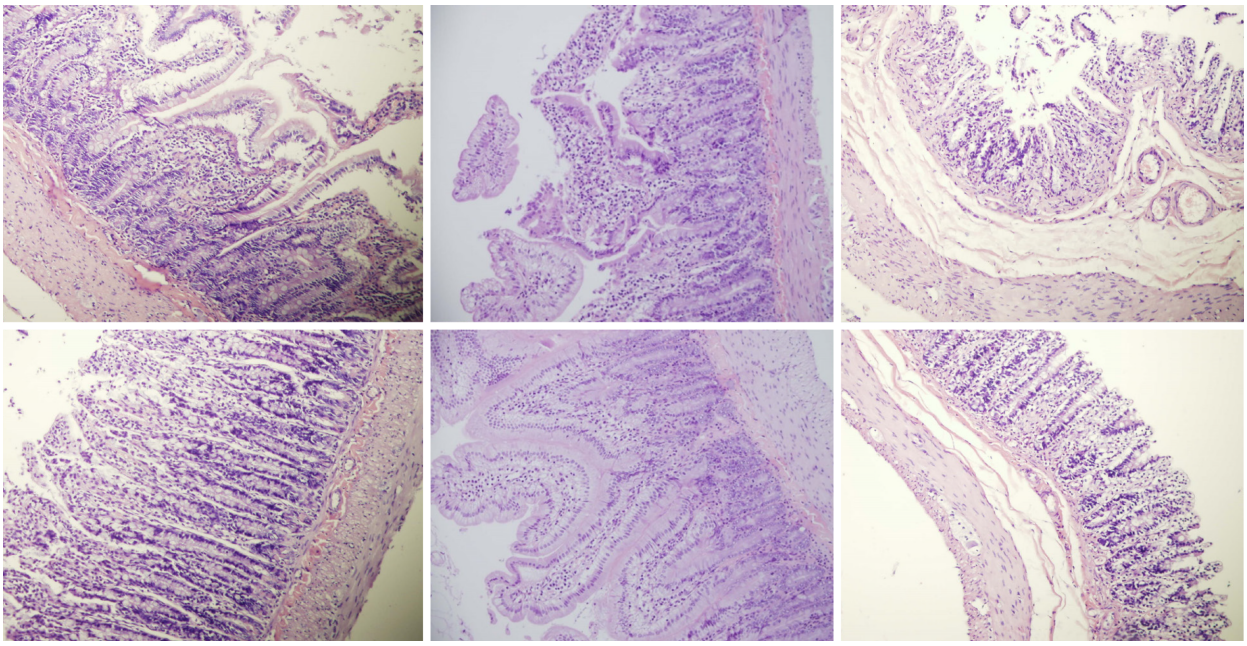

Figure 11 Histological presentation in rats with suprahepatic ligation of the inferior vena cava. Assessment (stained with hematoxylin-eosin, magnification x 10, scare bar: 50 μm) waste 48 h post-ligation, in controls (upper) and BPC 157-treated rats (lower), jejunum (left upper, control; lower, BPC 157), ileum (middle upper, control; lower, BPC 157), cecum (right upper, control; lower, BPC 157). Note, substantial capillary congestion with mild ischemic changes, loss of crypts with foci of haemorrhage, edema of the lamina propria and mild lymphocytic infiltration in controls (upper), which were markedly counteracted in BPC 157-treated rats (lower).

ARTICLE HIGHLIGHTS

Research background

Resolving the Budd-Chiari syndrome (BCS) in rats follows the evidence that pentadecapeptide BPC 157 therapy alleviated various vascular occlusion disturbances. Rapid activation of a bypassing loop rescues rats with inferior vena cava infrarenal ligation, relieving a Virchow's triad situation, much like in rats with ischemic/reperfusion colitis, duodenal venous congestion lesions, perforated cecum, bile duct ligation induced liver cirrhosis and portal hypertension. BPC 157 was previously reviewed as novel mediator of Robert cytoprotection and endothelium protection in the stomach, and gut-brain axis, beneficial therapy in gastrointestinal tract, with particular reference to vascular recruitment, ulcerative colitis and tumor cachexia, and other tissues healing.

Research motivation

Against these obstructions, BPC 157 therapy rapidly activates an azygos/hemiazygos vein bypassing pathway, upgrading an inadequate rescuing inferior-superior vena cava shunt to an adequate one, as well as a portocaval shunt. With BCS in rats, both caval and portal hypertension, and aortal hypertension occurred and were largely eliminated by BPC 157 therapy. Likewise, BPC 157 therapy was shown in counteracting rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST-elevation, tachycardia, gross organ lesions, and liver and spleen weight increases.

Research objectives

Occluding the inferior vena cava cranially to the hepatic veins, regardless of some limitations, was used for the Budd–Chiari syndrome and its manifestation in rat. To make the even more severe circumstances, with the complete occlusion of the suprahepatic inferior caval vein, Budd–Chiari syndrome has abrupt disturbances initiation. Thereby, BPC 157 rapid effect with the organized bypassing of the occlusion and Budd–Chiari syndrome manifestation reversal, may provide pharmacotherapy treatment in the rat BCS.

Research methods

In rats with occluded suprahepatic inferior caval vein, we assessed the activated bypassing pathways (inferior/superior caval veins; portocaval shunt), counteracted caval/portal hypertension, aortal hypotension, venous/arterial thrombosis, electrocardiogram disturbances, liver and gastrointestinal lesions (*i.e.*, stomach and duodenum hemorrhages, in particular, congestion), hepatomegaly and splenomegaly. Medication (at 1 min, 15 min, 24 h, or 48 h) ligation time consisted of 10 μg/kg BPC 157, 10 ng BPC 157 or 5 mL/kg saline, administered once as an abdominal bath or intragastric application. Furthermore, levels of nitric oxide (NO), malondialdehyde (MDA) in the liver and serum enzymes were determined.

Research results

The bypassing pathways (the inferior vena cava-azygos (hemiazygos) vein-superior vena cava; portocaval shunt) occurred rapidly. BPC 157 antagonized portal/caval hypertension (caval portal hypertension), and aortal hypotension, refractory ascites and thrombosis (portal vein tributaries, inferior vena cava, hepatic and coronary arteries), pathology of the lungs (severe capillary congestion) and liver (dilated central veins and terminal portal venules), intestine hemorrhagic lesions (substantial capillary congestion, submucosal edema and architecture loss), increased liver and spleen weight serum and enzymes values, NO- and MDA-levels in the liver. BPC 157 counteracted increased P wave amplitude, tachycardia and ST-elevation, *i.e.*, right heart failure from acute thrombotic coronary occlusion.

Research conclusions

Particular reversal of the regular threatening course otherwise arising in the rats with the occluded suprahepatic inferior caval vein. With application of the stable gastric pentadecapeptide BPC 157, we raised new hypothesis about rapid bypassing of the suprahepatic inferior caval vein occlusion in the BCS in rats, and mitigating its manifestations, along with the active and effective pharmacotherapy treatment. The beneficial BPC 157 effects, noted in the present study, may together counteract a downward spiral in rats with BCS, in acute states, much as in prolonged ischemia. In BCS research, rats studies used occluding the inferior vena cava cranially to the hepatic veins, but bypassing of the occlusion in the rat BCS along with pharmacotherapy treatment was not considered. BPC 157 therapy significance results with the rapidly activated azygos/hemiazygos vein bypassing pathway, upgrading an inadequate rescuing inferior-superior vena cava shunt to an adequate one, as well as a portocaval shunt. Consequently, the BCS-rats presented both caval and portal hypertension, and aortal hypertension, largely eliminated by BPC 157 therapy. Largely attenuated consequent disturbances (rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST-elevation, tachycardia, gross organ lesions, and liver and spleen weight increases) all together support this contention. We used gross (USB camera) and microscopic observations, venography, blood pressure and electrocardiogram assessment, bilirubin and enzyme activity, levels of NO, MDA in the liver and serum enzymes assessment. As combined methods, they are together new methods to determine description of the Budd Chiari syndrome in rats, and the significance of the activated bypassing pathways between the inferior and superior caval veins and portocaval shunt, counteracted caval/portal hypertension, aortal hypotension, venous/arterial thrombosis, electrocardiogram disturbances, liver and gastrointestinal lesions (*i.e.*, stomach and duodenum hemorrhages, in particular, congestion), hepatomegaly and splenomegaly. We should emphasize the rapidly activated azygos/hemiazygos vein bypassing pathway presented as an adequate rescuing inferior-superior vena cava shunt along with a portocaval shunt, both caval and portal hypertension, and aortal hypertension largely eliminated. As mentioned above, largely attenuated consequent disturbances (rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST-elevation, tachycardia, gross organ lesions, and liver and spleen weight increases) all together support this contention. Recently, as a possible therapy resolving solution for Budd Chiari syndrome in rats, pentadecapeptide BPC 157 therapy has been used in alleviating vascular occlusion disturbances. Rapid activation of a bypassing loop occurs in rats with an infrarenal ligation of the inferior vena cava, relieving a Virchow's triad situation, much like in rats with ischemic/reperfusion colitis, duodenal venous congestion lesions, perforated cecum, bile duct ligation induced liver cirrhosis and portal hypertension. BPC 157 therapy (intragastric or abdominal bath, 10 μg/kg and 10 ng/kg dosages), using a previously described protocol, could alleviate suprahepatic occlusion of the inferior caval vein, as model of the BCS in rats, activating bypassing pathways. BPC 157 likely counteracted Virchow's triad, as it did in rats with infrarenal inferior vena cava occlusion. A particular effect on portal hypertension disturbances, by activation of bypassing pathways, might be envisaged that needs further elaboration.

Research perspectives

The bypassing pathway of the inferior vena cava-azygos (hemiazygos) vein-superior vena cava and portocaval shunt occurred rapidly. Even with severe caval/portal hypertension, BPC 157 antagonized portal and caval hypertension and aortal hypotension. Large extent of the consequent disturbances may verify the significance of the Budd Chiari syndrome in rats for the corresponding human condition. Likewise, the largely attenuated disturbances may indicate that the beneficial BPC 157 effects, noted in the present study, may likely counteract a downward spiral in the rats with the suprahepatic inferior caval vein ligation much like in the patients with BCS, as in prolonged ischemia. To further establish the proposed therapy solution for Budd Chiari syndrome, we continue research in this issue. Gross (USB camera) and microscopic observations, venography, blood pressure and electrocardiogram assessment, bilirubin and enzyme activity, levels of NO, MDA in the liver and serum enzymes as combined methods are best methods to determine description of the Budd Chiari syndrome in rats.

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REVIEW

Pancreatic exocrine insufficiency after pancreaticoduodenectomy: Current evidence and management

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Abstract

Pancreaticoduodenectomy (PD) is the commonest procedure performed for pancreatic cancer. Pancreatic exocrine insufficiency (PEI) may be caused or exacerbated by surgery and remains underdiagnosed and undertreated. The aim of this review was to ascertain the incidence of PEI, its consequences and management in the setting of PD for indications other than chronic pancreatitis. A literature search of databases (MEDLINE, EMBASE, Cochrane and Scopus) was carried out with the MeSH terms "pancreatic exocrine insufficiency" and "Pancreaticoduodenectomy". Studies that analysed PEI and its complications in the setting of PD for malignant and benign disease were included. Studies reporting PEI in the setting of PD for chronic pancreatitis, conference abstracts and reviews were excluded. The incidence of PEI approached 100% following PD in some series. The pre-operative incidence varied depending on the characteristics of the patient cohort and it was higher (46%-93%) in series where pancreatic cancer was the predominant indication for surgery. Variability was also recorded with regards to the method used for the diagnosis and evaluation of pancreatic function and malabsorption. Pancreatic enzyme replacement therapy is the mainstay of the management. PEI is common and remains undertreated after PD. Future studies are required for the identification of a well-tolerated, reliable and reproducible diagnostic test in this setting.

Key words: Pancreatic exocrine insufficiency; Pancreaticoduodenectomy; Pancreatic enzyme replacement therapy; Pancreatic cancer; Malabsorption; Steatorrhoea

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Core tip: Pancreatic exocrine insufficiency is highly prevalent after pancreaticoduodenectomy and has significant implications to patients' quality of life, nutrition, post-operative survival and cancer related outcomes. The published literature reveals no uniform definition of pancreatic exocrine insufficiency after surgery and patients are often under diagnosed. Pancreatic enzyme replacement therapy is effective, well tolerated and is indicated routinely in this cohort of patients. Future studies need to focus on the identification of a well-tolerated, reliable and reproducible diagnostic test in this setting that will facilitate a uniform definition and management approach.

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INTRODUCTION

Pancreatic enzymes are an essential component of normal digestion, without which severe malnutrition occurs. Nonetheless, pancreatic exocrine insufficiency remains widely under-diagnosed and undertreated. The physiological secretion of pancreatic enzymes is in response to nutritional intake in healthy individuals. The stimulation occurs through three phases: Cephalic, gastric and the most important intestinal phase[1]. The pancreatic enzyme secretion peaks at about 30 min after the exposure of the duodenum to nutrients and returns to baseline after about 2-4 h. The presence of undigested food, especially fat, in the terminal ileum exerts a robust negative feedback mechanism[2-7].

Pancreatic exocrine insufficiency (PEI) is a common and recognized outcome after pancreatic surgery. Multiple definitions have been used in the published literature based on various evaluation parameters. The "broadest" definition was presented in a systematic review by the Spanish pancreatic association and defined PEI as the inability of the pancreas to perform digestion in association with disturbed pancreatic function[8].

Pancreaticoduodenectomy (PD) is an operative procedure that involves resection of the pancreatic head in addition to the duodenum and bile duct. It is the most common pancreatic resection performed, especially in the setting of pancreatic malignancy. The effect of PD on pancreatic exocrine secretion is multifactorial. The degree of insufficiency is influenced by the pancreatic remnant[9], preservation or resection of the gastric antrum and duodenum[10], the use of a roux-en-Y loop with asynchrony of delivery of the pancreatic enzymes[11,12] and other factors, such as the peri-operative use of Octreotide[13]. In the context of pancreatic surgery, PEI has been associated with prolonged hospital stay[14], increased complication rates[15], reduced survival[16], worse quality of life[8] and nutritional deficiencies[17]. Furthermore, the presence of PEI may also impede the progression of patients to adjuvant chemotherapy in the setting of resections performed for malignancy.

The purpose of this paper is a comprehensive review of the current evidence on the incidence and management of PEI specifically in the setting of PD for indications other than chronic pancreatitis.

STUDY SELECTION

The intention was to proceed with a systematic review of the incidence and management of PEI in the setting of PD. Studies were selected in accordance with Preferred Reporting of Systematic Reviews and Meta-analyses (PRISMA) 2009 guidelines[18].

A literature search of databases (MEDLINE, EMBASE, Cochrane and Scopus) was carried out by two separate authors (AP and JA). The search was constructed by using the Medical Subject Heading (MeSH) terms "pancreatic exocrine insufficiency" and "Pancreaticoduodenectomy". Studies that analysed the incidence of PEI in the setting of PD were included. Studies that focused on complications of PEI after PD were also

included. Case reports, reviews, consensus statements, conference abstracts and articles in languages other than English were excluded. Studies on pancreatic resections for chronic pancreatitis were excluded, as well as studies that did not sub-classify patients according to the type of pancreatic resection and therefore data on PEI after PD could not be extracted.

The search led to a total of 746 hits. After removal of duplicates and articles in languages other than English, 556 articles remained. Further screening and full text review of articles resulted in a total of 34 articles eligible for inclusion in the review. The steps of the selection process are collated in Figure 1.

APPRAISAL OF LITERATURE

An attempt at data extraction revealed that studies used different parameters to define PEI as explained below in the results. This meant that a quantitative analysis of the results was not possible. Narrowing down studies further with stricter inclusion criteria meant that a large body of evidence would be left out of the analysis thereby subjecting the review to significant bias. A recent systematic review on the same subject, which included a total of only 9 studies, highlighted this aspect[19]. It was therefore decided to proceed with a qualitative narrative review of the subject.

DEFINITION OF PANCREATIC EXOCRINE INSUFFICIENCY

In chronic pancreatitis, PEI is defined by the presence of steatorrhoea and commonly assessed by the concentration of faecal elastase-1 (FE-1) in a random stool sample[20]. In this setting, FE-1 is known to reflect the level of pancreatic function and water reabsorption in the gastrointestinal tract[21,22]. It has been validated and correlates well with radiological findings and steatorrhoea in chronic pancreatitis[23-26]. FE-1 in the setting of chronic pancreatitis has also been used to grade the severity of PEI (Normal- > 200 μg/g stool; mildly impaired - 100-200 μg/g stool and severe - < 100 μg/g stool)[27].

Following pancreatic surgery, however, there is no consistent definition for PEI. Furthermore, various diagnostic tests have been used in this setting, while the accuracy of FE-1 is reduced making it an unreliable test. Table 1 highlights some of the most common parameters used to define PEI in patients undergoing pancreatic surgery and especially PD[28-34].

PARAMETERS FOR THE CLINICAL ASSESSMENT OF PANCREATIC EXOCRINE INSUFFICIENCY FOLLOWING PANCREATICODUODENECTOMY

The most characteristic clinical presentation of PEI is steatorrhoea, defined as the presence of more than 7 g of stool fat/day[35]. However, steatorrhoea is a late sign and associated with severe PEI (occurring after a loss of more than 90% of pancreatic function). Therefore, a methodical diagnostic approach is warranted, including complete medical and dietetic history, physical examination and serial anthropometric measurements, supplemented by biochemical tests and in some scenarios by relevant imaging investigations[36].

Due to the low diagnostic sensitivity of steatorrhoea, other PEI-related (but also not specific) symptomatology is important. A history of flatulence, bloating, urgency and abdominal discomfort or post-prandial abdominal pain may assist in the diagnosis of PEI. PEI is also associated with weight loss and reduction in muscle mass[10,37]. Other symptoms such as nausea, early satiety, vomiting, oral thrush and ulcers (secondary to concurrent chemotherapy) may adversely affect the dietary intake contributing to malnutrition in these patients. Dietary modifications (consciously or subconsciously by the patients), such as restriction of protein and/or fat intake, may result in masking the symptomatology, including steatorrhoea, and therefore lead to late or misdiagnosis[17,36].

A previous history of endocrine disorders (importantly diabetes mellitus), bowel conditions (such as coeliac disease, irritable bowel syndrome *etc.*), food intolerances or eating disorders is relevant. Previous surgery to the bowel (*e.g.* gastrectomy, small bowel resection, and colectomy) can also affect the gut function and alter microbiota causing symptoms that may aggravate or mimic PEI. Drugs like probiotics, antibiotics, laxatives, anti-diarrhoea agents also influence gut function, while others,

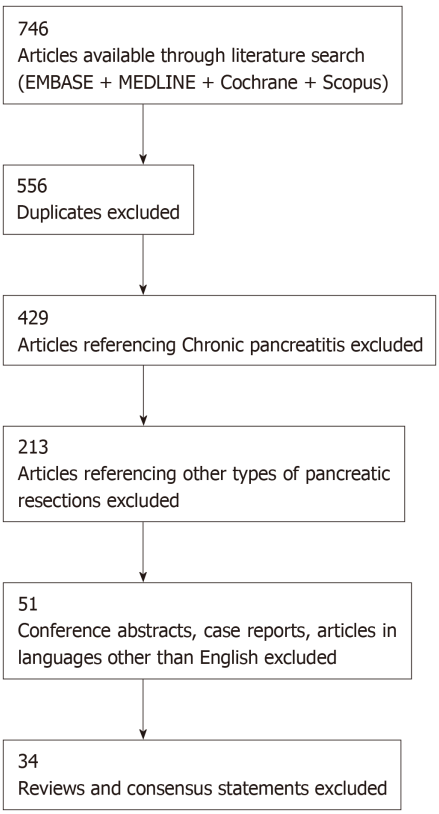

Figure 1 Literature review and selection process.

such as steroids and insulin, can also have an additional impact on the patient's weight in addition to affecting gut absorption[36]. Serial anthropometric measurements are invaluable to monitoring the nutritional status and important to assess the response to therapeutic interventions. Functional assessments, such as grip strength, mid arm circumference and triceps skin fold, together with weight changes, must be evaluated in the context of the patient's symptoms and caloric intake.

BIOCHEMICAL PARAMETERS FOR THE ASSESSMENT OF PANCREATIC EXOCRINE INSUFFICIENCY

Relevant laboratory investigations fall into two main categories: (1) Evaluation of the nutritional status, and (2) Evaluation of the pancreatic function (Table 2). The first category includes tests such as the assessment of fat soluble vitamins, bone profile (calcium, parathyroid hormone), anaemia screen and glycaemic control. These can be used for the initial diagnosis, as well as for follow-up and evaluation of the treatment response. The second category includes tests that evaluate the pancreatic function and are further broadly sub-classified into those that evaluate the exocrine function of the pancreas and tests that measure the degree of malabsorption secondary to PEI. The latter ones focus mainly on fat malabsorption with the limitation that they cannot distinguish between pancreatic and extra-pancreatic causes. Currently there are no tests available to diagnose nitrogen malabsorption also known to occur in PEI[38], while colonic mechanisms exist to compensate for the malabsorption of carbohydrates[39,40].

The 2018 ISGPS position statement considered 72 h faecal fat collection with a standard intake of fat as the gold standard test to diagnose fat malabsorption[17]. FE-1 measurement is one of the most commonly used methods to evaluate and subsequently define PEI. It is quick, non-invasive, and relatively easy to carry out in the clinical setting (on a spot faecal sample). Additionally, it is not influenced by the intake of pancreatic enzyme supplements. However, in the setting of PD, steatorrhoea occurs at a much higher FE-1 level (207 μg/g in patients post PD *vs* 15 μg/g in patients without a resection)[41], therefore its usefulness in this setting is questionable. Kato *et al*[32] detected PEI in 93% patients prior to PD (most of which with a diagnosis of pancreatic cancer) on the basis of the secretin stimulation test. The comparison of

Table 1 Definitions of Pancreatic Exocrine Insufficiency after pancreaticoduodenectomy

Ref.	Definition of pancreatic exocrine insufficiency
Sabater *et al*[8]	Condition wherein the amount of pancreatic secretions is not enough to maintain normal digestion
Ghaneh *et al*[28]	Need for new pharmacological intervention for exocrine insufficiency *i.e.* PERT
Sikkens *et al*[11]	Faecal elastase-1 < 0.200 mg/g of faeces
Halloran *et al*[29]	Coefficient of fat absorption < 93%
Domínguez-Muñoz *et al*[30]	^{13}C-mixed triglyceride test (Percent cumulative dose of < 5% of $^{13}CO_2$ at 7 h)
Yamaguchi *et al*[31]	BT-PABA excretion rate of < 70%
Kato *et al*[32]	Abnormal secretin stimulation test
Perez *et al*[33]	72 h faecal fat estimation
Fang *et al*[34]	Faecal chymotrypsin estimation

PERT: Pancreatic enzyme replacement therapy.

this test was with ^{13}C- labelled trioctanoin breath assay and with parallel testing of para-aminobenzoic acid (PABA) and faecal chymotrypsin excretion. The sensitivities of both these tests were between 60% and 70% in the setting of obstructive jaundice and PD[32]. The current review thus, reveals a lack of consensus on the parameters used to evaluate PEI after PD.

INCIDENCE OF PANCREATIC EXOCRINE INSUFFICIENCY IN THE SETTING OF PANCREATICODUODENECTOMY

Due to the nature of this review, studies reporting outcomes among patients undergoing PD for chronic pancreatitis were excluded. The reported incidence of PEI after PD varied widely between 38% and 93% (Table 3)[42-55]. This is probably attributed to the heterogeneity of the patient cohorts and the diagnostic tests used.

Halloran *et al*[29]showed an improvement in FE-1 after PD for pancreatic cancer, however, this was in the setting of a diminishing patient cohort (exclusion of patients with mortality)introducing the possibility of bias. Additionally, FE-1 did not compare accurately to the standard measure of PEI (Coefficient of Fat absorption). Other studies have consistently recorded improving pancreatic function in patients with ampullary cancer post-PD[56,57]. The proposed hypothesis in these studies was the relief of the obstruction by the ampullary tumour to the pancreatic duct draining a healthy pancreas.

The correlation of pre-operative PEI to post-operative PEI is difficult to assess as FE-1 is the most frequently used marker and has been shown to underestimate PEI after pancreatic resection[19]. Matsumoto *et al*[47] noted a significant post-operative drop in FE-1 levels in patients with normal pre-operative values, while FE-1 levels in those with pre-existing PEI remained relatively unchanged post-operatively. It is possible that these findings are limited not only by the use of FE-1 in post-operative assessment, but also by the short follow-up period. This is further supported by the diagnosis of PEI in all patients at a median post-operative time of 52 mo[58].

There are several studies that have investigated possible predictors of PEI after PD, such as the presence of a dilated pancreatic duct on computerized tomography (CT) scans or endoscopic ultrasound pre-operatively[59]. One study reported that a dilated pre-operative duct diameter (> 3 mm) was more likely to result in exocrine dysfunction at 2 mo after surgery measured by reduced PABA excretion[45]. This finding was however, not corroborated by Matsumoto *et al*[47] who suggested that the diminishing pancreatic parenchyma was the main reason for the reduced post-operative FE-1 levels. Furthermore, post-operative parenchymal thickness on CT was shown to be a predictor of PEI (based on the ^{13}C-labelled mixed triglyceride test) with a sensitivity of 88.2% and specificity of 88.9% when the cut off was set at 13 mm[60]. Nonetheless, the use of imaging findings to clinically predict PEI remains in use predominantly in the setting of chronic pancreatitis[25,61,62].

Table 2 Biochemical tests in the assessment of pancreatic exocrine insufficiency

Nutritional assessment	Evaluation of pancreatic function	
	Exocrine markers	Markers of malabsorption
Fat soluble vitamins	Faecal elastase-1	72 h faecal fat estimation
Bone profile	Faecal chymotrypsin	BT-PABA absorption
Iron and Ferritin studies	Secretin stimulation test	^{13}C labelled trioctanoin breath test
Micronutrient status		
Glycaemic status		

PABA: Para-aminobenzoic acid.

TECHNICAL OPERATIVE FACTORS INFLUENCING PANCREATIC EXOCRINE INSUFFICIENCY

The pre- and post-operative incidence of PEI was studied with a BT-PABA test in patients undergoing classical PD versus Pylorus preserving PD (PPPD). The short term post-operative incidence was similar in both groups. The exocrine function recovered to pre-operative levels in the PPPD group, while this was not observed in the classical PD group. The study, however, was limited by the small patient cohort (10 classical PD *vs* 44 PPPD) and the potential for selection bias across the two groups, while the indications included both benign and malignant diagnoses[45].

The effect of the type of reconstruction, pancreatico-gastrostomy or pancreatico-jejunostomy, on PEI has also been studied (Table 4). Two retrospective studies reported that patients undergoing pancreatico-jejunostomy reconstruction for pancreatic head malignancy were significantly less likely to have PEI[54,63]. Others have also shown a similarly high incidence of PEI after pancreatico-gastrostomy in retrospective cohorts[9,44]. However, the retrospective comparative study by Jang *et al*[51] showed no significant difference between the two reconstruction methods (100% *vs* 95%). This conflicting evidence is most likely attributed to the use of different methods to measure and report the incidence of PEI, including 72 h faecal fat estimation, ^{13}C-labelled mixed triglyceride breath test and measurement of FE-1.

CONSEQUENCES OF PANCREATIC EXOCRINE INSUFFICIENCY AFTER PANCREATICODUODENECTOMY

In the perioperative setting, PEI can lead to malnutrition and this in turn to higher morbidity and mortality including a greater risk of a pancreatic leak[59,64,65]. Additionally, it may significantly affect quality of life and it has been shown to be an independent predictor of survival in advanced pancreatic cancer[66]. Similarly, cachexia, has shown to be associated with decreased survival with unresectable pancreatic cancer, with weight stabilization showing better prognosis[67-69].

There is increasing evidence that untreated PEI negatively affects survival following PD for cancer. Among consecutive patients undergoing PD for periampullary cancer those without treatment had significantly reduced survival; this was even more pronounced among the cohort with pancreatic duct dilation (≥ 3 mm)[70]. A further population based study used propensity matched analysis to adjust for key variables and in that study lack of treatment of PEI was associated with reduced survival and the survival benefit of pancreatic enzyme replacement therapy (PERT) was of a similar magnitude to surgery or chemotherapy[71].

The symptoms and consequences of PEI after PD are mainly related to the malabsorption of undigested food and nutrients, especially fat soluble vitamins (Vitamins A, D, E and K)[17] in the distal small bowel[72]. The classical symptoms of steatorrhoea, abdominal pain with bloating and cramping, flatulence, dyspepsia and nausea are however, not seen in patients with mild to moderate PEI[73]. Vitamin malabsorption may lead to symptoms such as xerophthalmia and night blindness (Vitamin A), neurological symptoms, ophthalmoplegia and ptosis (Vitamin E), abnormal bleeding (Vitamin K), osteomalacia and metabolic bone disease (Vitamin D). It is important to recognize these as potential complications of PEI early and start supplementation (parenterally if necessary) on a long term basis. Other complications such as weight loss, electrolyte imbalances and poor wound healing may also occur[17]. In cases where the indication for PD is cancer, malnutrition can delay the start of the

Table 3 Incidence of pancreatic exocrine insufficiency before and after pancreaticoduodenectomy

Ref.	Pre-operative incidence of PEI	Post-operative incidence of PEI	Diagnostic test
Kato *et al*[32]	93%	80%	Secretin stimulation
Halloran *et al*[29]	-	55%	Coefficient of fat absorption
Yuasa *et al*[42]	-	64%	^{13}C- mixed triglyceride test
Nakamura *et al*[9]	-	62.3%	
Hirono *et al*[43]	-	51%	
Benini *et al*[41]	-	87.5%	72 h faecal fat estimation
Lemaire *et al*[44]	-	94%	
Sato *et al*[45]	46%	33%	BT-PABA excretion
Fujino *et al*[46]	-	75%	
Matsumoto *et al*[47]	68%	50%	Faecal elastase-1
Van der Gaag *et al*[48]	-	59%	
Tran *et al*[49]	-	91%	
Pessaux *et al*[50]	-	95%	
Jang *et al*[51]	-	100%	
Falconi *et al*[52]	-	24%	Faecal chymotrypsin
Fang *et al*[34]	-	33%	
Bock *et al*[53]	-	52.8%	Steatorrhoea
Rault *et al*[54]	-	42%	
Van Berge Henegouwen *et al*[55]	-	64.5%	

PEI: Pancreatic exocrine insufficiency; PABA: Para-aminobenzoic acid.

recommended adjuvant chemotherapy or worse, render the patient unfit for the same. Finally, NAFLD is a rare and poorly recognized possible consequence of PEI after PD. It is believed to occur secondary to the malabsorption of essential amino acids leading to decreased plasma levels of apoprotein B[74], which, when combined with sub-optimal insulin secretion lead to peripheral lipolysis and greater hepatic fat deposition[75]. These changes have been shown to be reversible with the administration of PERT and subsequent improvements in body weight[76].

MANAGEMENT OF PANCREATIC EXOCRINE INSUFFICIENCY AFTER PANCREATICODUODENECTOMY

PERT is the mainstay of treatment of PEI. However, in the post-operative setting, there is a lack of consensus over the timing of initiation of PERT. While some authors recommend routine post-operative PERT[36,77], others advocate in favour of PERT only after clinical or biochemical diagnostic evidence of PEI[8,78]. In pancreatic cancer, due to the high incidence of PEI and obstructive jaundice, peri-operative use of PERT for all patients has been shown to be beneficial[36] and is recommended by the United Kingdom National Institute of Clinical Excellence guidelines[79].

Patient education is important for the correct use of PERT. Enzymes use is advisable with all meals, snacks and milky drinks, including various supplements. The conventional timing of administration is during or immediately after a meal in order to achieve optimal timing for mixing with the chyme. This hypothesis however, has not been studied in the setting of pancreatic surgery and the presence of pancreatico-biliary reconstruction and digestive asynchrony[16].

PERT is usually commenced at a dose of 50000-75000 units lipase with a meal and 25000-50000 units with each snack[10,80-82]. This may be titrated to the needs of the individual patient. In common clinical experience, patients over time learn to adjust the dose of PERT on the basis of their symptoms and diet. Nonetheless, close follow-up is required to ensure that management remains on track in the setting of changing (recovering or deteriorating) pancreatic function and/or patient diet (as some patients may compromise on the nutritional value of their diet rather than the PERT dose). The use and effectiveness of PEI should be monitored with serial anthropometric measurements and nutritional blood tests[36], including measurements for glycaemic control, as the use of effective PERT may result in manifestation of diabetes[83]. In addition to PERT, supplementation with vitamins and other micronutrients is

Table 4 Incidence of pancreatic exocrine insufficiency after pancreaticoduodenectomy–evidence on the role of the type of pancreatic reconstruction

Ref.	Diagnostic test	Incidence of PEI–Pancreaticogastrostomy	Incidence of PEI–Pancreaticojejunostomy
Nakamura *et al*[9]	^{13}C Triglyceride breath test	62.3%	-
Lemaire *et al*[44]	Faecal Fat excretion and faecal elastase-1	100%	-
Jang *et al*[51]	Faecal elastase-1	100% (severe)	75% (severe); 20% (mild)
Roeyen *et al*[63]	Need for PERT +/- any abnormal pancreatic function test	75%	45.7% ($P < 0.001$)
Rault *et al*[54]	Steatorrhoea	70%	21.7% ($P < 0.025$)

PEI: Pancreatic exocrine insufficiency; PERT: Pancreatic enzyme replacement therapy.

recommended[84].

The gastrointestinal environment and acidity is important for the appropriate function of PERT. The lipase in PERT is inactivated by gastric acid activity. Consequently, commercially available PERT formulations are covered with pH sensitive, acid resistant microspheres that release the lipase at a pH of 5-6, similar to what is present in the native duodenum. Based on studies about the optimal sphere size required to produce the best dissociation in the duodenum, most commercial preparations have sphere size that varies from 1-2 mm[85,86]. In the post-operative PD setting, failure of the pancreas to produce bicarbonate is hypothesized to lead to an acidic environment in the duodenum and proximal jejunum, leading to inefficient activation of lipase[35,87]. The concurrent use of gastric acid suppression is therefore recommended. The use of a proton pump inhibitor is known to reduce faecal fat losses[88] and may also help reduce precipitation of bile salts[36].

PERT is generally well tolerated with minimal adverse effects. Rare reports on fibrosing colonopathy with the use of PERT are limited to paediatric patients, especially in the setting of cystic fibrosis[89-91]. There have been no such reports in the adult post-operative population. Many studies including open label PERT trials have not found significant adverse drug reactions[92-94].

Failure of PEI to improve after escalation of PERT dosage and gastric acid suppression must prompt further investigations for concurrent problems. The two commonest diagnoses in this setting are bile salt malabsorption and small bowel bacterial overgrowth[30,80,82]. Bile salt malabsorption occurs due to the change in the pH in the proximal small bowel secondary to deficiency of bicarbonate secretion from the pancreas. The cholecystectomy performed during PD may also contribute to the development of this condition[36,95]. The presence of a blind loop of bowel used for reconstruction is known to occur after PD and is documented in up to 65% of patients leading to small bowel bacterial overgrowth[36].

CONCLUSION

This literature review confirms that PEI is prevalent after PD even for indications other than chronic pancreatitis and may have severe implications with respect to patients' survival, quality of life, nutrition and subsequent management. The lack of a uniform definition of PEI in this setting and the low diagnostic accuracy of the available tests introduce a wide variability in the reported results and suggested management. Pancreatic enzyme replacement therapy is effective, well tolerated and is indicated routinely in this cohort of patients. Future studies need to concentrate on the identification of a well-tolerated, reliable and reproducible diagnostic test that will facilitate a uniform definition and management approach.

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ORIGINAL ARTICLE

Observational Study

Outcomes of a drug shortage requiring switching in patients with ulcerative colitis

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Abstract

BACKGROUND

Drug shortages are common yet their impact on patient care and their commercial ramifications has not been adequately researched. In Australia a shortage of balsalazide (2012-2013) necessitated substitution with alternative 5-aminosalicylate (5-ASA) formulations for ulcerative colitis (UC).

AIM

To assess and compare the clinical and commercial sequelae of non-medical switching from balsalazide to another 5-ASA and/or return to balsalazide once supply resumed.

METHODS

A prospective cohort study of patients on balsalazide for mild-moderate UC was conducted where, strictly due to the national shortage (November 2012- January 2013), were switched to alternative 5-ASA and/or then returned to balsalazide once supply resumed. Clinical (Partial Mayo), endoscopic (Mayo score) activity, adverse effects (to alternative 5-ASA) and percentage market share (of continuous 5-ASA users) from baseline (*i.e.*, time of switching due to shortage) through to five years were assessed.

RESULTS

Of 31 patients switched due to the shortage, 12 (38.7%) resumed balsalazide immediately once supply resumed, 8 (25.8%) prompted by adverse effects to the

alternative 5-ASA used. Three patients (9.7%) had documented symptomatic improvement, 15 (48.4%) were unchanged and 13 (41.9%) had symptomatic worsening *vs* baseline ($P < 0.01$), after switching to an alternative 5-ASA. At 3 and 5y post switch, overall 26/31 (83.9%) and 23/31 (74.2%) had remained continuously on any 5-ASA therapy respectively. Twelve (38.7%) and 11 (35.5%) patients remained on balsalazide continuously at three and five years respectively after drug supply returned, equating to a loss of market share (within 5-ASA class) of 45.2% and 38.7% respectively.

CONCLUSION

This study of a balsalazide shortage in UC patients exemplifies the detrimental impact of a drug shortage on long term patient, disease and commercial outcomes.

Key words: Inflammatory bowel disease; Ulcerative colitis; Drug supply; Drug shortage; Patient outcomes; Market share

Core tip: As a chronic disease, this study of a drug shortage in ulcerative colitis provides an excellent, novel insight into the short and long term effects of an sudden, unexpected nationwide drug shortage (in this case balsalazide) in patients previously in stable remission. The study highlights the importance of maintaining a seamless drug supply for both patients (given significant rates of disease worsening occurred, directly attributable to shortage), and drug manufacturers given the loss of market share engendered by even a short term drug shortage.

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INTRODUCTION

Drug supply shortages are an insidious yet growing threat to the optimal medical management of chronic diseases, including ulcerative colitis (UC). Across many countries, healthcare settings and diseases, such shortages have been linked with patient deaths and significant morbidity[1,2]. Drug shortages may be defined by "a supply issue that affects the ability of preparation and/or dispensing of a drug such that it influences patient care and/or prescribers must use an alternative agent[3]". They appear to be increasing for multiple reasons, including growing trends worldwide to operate drug supply chains on a just-in-time basis to maximise cost reduction and avoid excess inventory, brought about by lower profit margins due to the arrival of generics and other market competitive forces. Moreover, in most countries, there is no requirement for suppliers to report drug supply issues, often leading to clinicians and patients facing shortages with little or no warning. High cost drugs for relatively narrow indications in smaller markets appear more at risk of shortages, exemplified by the balsalazide shortage in Australia as studied here[4-6].

Over a three month period from November 2012 to January 2013 inclusive, a nationwide shortage in the supply of balsalazide (*Colazal, Colazide*®) in Australia necessitated a blanket, non-medical substitution with alternative 5-ASA formulations in patients with UC. Balsalazide is a prodrug of the active metabolite, mesalazine, which is linked to a carrier molecule via an azo bond and released in the colon by bacterial azo-reduction[7]. The 5-aminosalicylic acid (5-ASA) agents as a class (including balsalazide) are standard and effective therapies for the induction and maintenance of remission in UC, particularly in mild to moderate disease. In the setting of few randomized head-to-head trials or real-world comparative studies of different oral 5-ASA preparations, the formulations are, pragmatically, considered to be similar in efficacy when adjusting for dose for both induction and maintenance of UC[8]. For instance, 6.75 g daily of balsalazide (equivalent to 2.34 g of mesalazine) was

compared with a pH-dependent acrylic resin coated mesalazine formulation (*Asacol*®) in three randomized-controlled, double-blind studies[9-11], with no significant differences in the primary endpoints between these formulations at 8 wk.

Therefore, this balsalazide shortage presented a unique opportunity to evaluate short and long term outcomes of a semi-controlled, unselected cohort of patients with UC in whom a non-medical switch in 5-ASA formulation was undertaken contemporaneously. Moreover, there are scant data regarding the short and long term implications of a drug shortage in patients with chronic diseases, including IBD. Hence, the aims of this prospective study were to assess the short and long term ramifications of a drug shortage imposed substitution of balsalazide to alternative 5-ASA formulations in patients with UC, in terms of: (1) Efficacy (including symptom-based, endoscopic activity and long-term outcomes); (2) Safety (including immediate adverse effects to alternative 5-ASA upon switching); and (3) The proportion of patients returning to the original product once supply resumed as a measure of loss of market share in a competitive drug category such as 5-ASA agents.

MATERIALS AND METHODS

A prospective, long term cohort study was undertaken of patients with a confirmed diagnosis of mild-moderate UC as per standard criteria, treated for a minimum of six months with balsalazide at specialist inflammatory bowel disease (IBD) Clinics at two Melbourne metropolitan hospitals, in whom switching to an alternative 5-ASA formulation (at the treating doctor's discretion) was mandated by drug unavailability between November 1st 2012 to January 31st 2013. Patients who were switched from balsalazide to an alternative 5-ASA due to medical reasons, including intolerance, tablet burden or active disease were excluded from this study. After supply of balsalazide resumed, the occurrence of switching back to balsalazide was solely at the discretion and agreement of the patient and treating clinician.

Patient demographics, disease characteristics (including Montreal Classification extent/severity of IBD[12]), biomarkers including C-reactive protein (CRP) and faecal calprotectin (where available), medication changes (including adverse effects and concomitant medications) and symptom-based disease activity (*via* partial Mayo score) were collected immediately at baseline pre-switch and then at multiple timepoints (3-6 mo at post-switch subsequent clinic review, then at 3 years and 5 years) after 5-ASA substitution. A balsalazide dose of 6-6.75 g was assumed to be equivalent to sulfasalazine or mesalazine 2-2.4 g as per the manufacturer's product information documentation.

Endoscopic data (*via* Mayo endoscopy score) were also collected at baseline (data included if endoscopy had occurred no more than 12 mo pre-switch), then post-switch (no less than 3 mo and no more than 12 mo from date of switch) and subsequently at three and five years (included if within six months in each case). A Mayo endoscopy score of 0 or 1 was deemed to represent endoscopic remission. Clinical remission was defined as a partial Mayo score of ≤ 2, where each subscore was ≤ 1. Patients with newly diagnosed ulcerative colitis in the preceding 12 mo prior November 1st 2012 were excluded from the analysis.

The impact on market share of balsalazide affected by the drug shortage was calculated as the proportion derived by the number of patients who were switched temporarily to an alternative 5-ASA before returning to balsalazide through to the three and five-year timepoints, divided by the total number of patients who, upon switching from balsalazide to an alternative 5-ASA, then remained on this or any alternative 5-ASA agent continuously to the respective timepoints.

Data were analyzed with GraphPad Prism version 7 (GraphPad software, La Jolla, United States 2018). Given the data were non-normally distributed and of relatively small sample size, non-parametric statistics were used. For continuous data, medians are presented and compared with Kruskal-Wallis (unpaired) or Wilcoxon (paired) tests. For categorical data, proportions were compared with Fisher exact tests. Patients without complete data at both baseline and five year follow-up timepoints were excluded from the study. A $P < 0.05$ was considered to be clinically significant. The statistical methods of this study were reviewed by Dr Danny Con, Biostatistician, Eastern Health. Ethics approval was obtained from the Eastern Health Office of Research and Ethics (LR 61/2015).

RESULTS

Thirty-one patients with UC were switched from balsalazide to an alternative 5-ASA

formulation specifically due to the balsalazide drug shortage. The majority of patients were switched from balsalazide to multi-matrix (MMX) mesalazine (28, 90.3%). Further characteristics of this cohort are shown in Table 1.

Short term outcomes post switch compared to baseline (pre-switch)

Compared to baseline clinical (partial Mayo score) activity, three patients (9.7%) had documented symptomatic improvement (≥ 1 point reduction in partial Mayo score), 15 (48.4%) were unchanged and 13 (41.9%) had symptomatic worsening (≥ 1 point increase in partial Mayo score) (improvement *vs* worsening, $P < 0.01$, Fisher exact test) after switching from balsalazide to an alternative 5-ASA formulation.

Twenty-six (83.9%) of the cohort had endoscopic assessment both within 12 mo prior and then within 12 mo after the switch. Of these, compared to baseline endoscopic activity (Mayo endoscopy score), 13 (50%) patients had similar or improved endoscopic activity and 13 (50%) had worsening of their endoscopic activity post switch ($P = 1.0$, Fisher exact test). There were no significant differences (pre to post-switch) in serum CRP [median difference 0 mg/L (-6, 108)], serum ALT [1.0 (-15, 61)], serum GGT [0.5 (-8, 507)] or serum white cell count [-0.5 (-3.0, 3.3)] (all tests +/−3 mo of switch, each $P > 0.2$, Wilcoxon tests).

Based on the published dose equivalence of balsalazide compared to mesalazine[7], in all 31 patients there was an equal or increased effective mesalazine dose received after switching to the alternative 5-ASA formulation [median delta increase of 1.4 g (0, 3.2) mesalazine daily, $P < 0.01$ (Wilcoxon test)], Figure 1.

Adverse events with substitution of balsalazide to alternative 5-ASA agent during shortage

Adverse events were reported by 8/31 (16.2%) patients, all documented within 2 wk after switching, and attributable to, the alternative 5-ASA agent. These included one or more of hepatotoxicity ($n = 2$), abdominal pain ($n = 6$), nausea ($n = 1$) and/or hypersensitivity reaction ($n = 1$). Of these patients with adverse events, all 8 (100%) had prompt resolution of symptoms upon cessation of alternative 5-ASA therapy. Then upon switching back to balsalazide once supply returned, all 8 continued on balsalazide without further adverse event/s during the remainder of the follow-up period (Figure 2).

Long term outcomes

At three and five years following the switch, overall 26/31 (83.9%) and 23/31 (74.2%) patients had remained continuously on any 5-ASA therapy respectively. Twelve (38.7%) patients switched back to balsalazide as soon as supply returned (within three months). All twelve (38.7%) remained on balsalazide at 3 years, with 11 patients (35.5%) on balsalazide at 5 years.

Compared to the total number and accounting for the resultant attrition of those on continuous 5-ASA therapy, there was a loss of long-term market share of 45.2% and 38.7% at three and five years respectively after, and as a direct result of, the balsalazide shortage (Figure 3). Also, at each subsequent timepoint following the switch through to five years, there were no significant differences in the rates of clinical or endoscopic remission between those who continued on alternative 5-ASA therapy *vs* those who had switched but then returned to balsalazide as soon as supply returned (Figure 4A-B).

Finally, there was no significant difference in rates of treatment escalation to immunomodulators or biologics, colectomy or mortality (all causes) between the two groups at both the 3 and 5-year follow-up timepoints. However, there was a higher rate of flares requiring hospitalization in those who switched from balsalazide then remained on an alternative 5-ASA (36.8 *vs* 0.0% at 5 years, $P = 0.03$, Fisher exact test), Table 2.

DISCUSSION

To our knowledge, this study is the first to demonstrate the long-term ramifications of a drug shortage in a cohort of patients with inflammatory bowel disease. Mild-moderate UC is an archetypal chronic disease in which to examine the effect of a drug shortage given it is a lifelong, relapsing-remitting disease in typically young, otherwise healthy patients, where remission is achieved and maintained in a significant proportion by daily administration of oral drugs such as mesalazine or balsalazide, with a highly favourable risk: benefit ratio. Long-term stability of disease control and outcomes depend primarily on adherence, and therefore continuous supply, of the drug. Consequently, a drug shortage has the potential to exert multiple deleterious flow-on effects including a flare or worsening of disease with possible

Table 1 Characteristics of the patient cohort (*n* = 31) who were switched from balsalazide (due to shortage) to an alternative aminosalicylate formulation

Variable	Pre-switch (baseline)	Post-switch (at subsequent review)[1]
Age (yr) (median, range)	54 (20-79)	
Male sex (%)	16 (51.6)	
Disease duration (yr) (median, range)	10 (3-48)	
Montreal Classification, *n* (%)		
Disease extent		
Proctitis (E1)	4 (12.9)	
Left sided colitis (E2)	21 (67.7)	
Extensive colitis (E3)	6 (19.4)	
Disease severity		
Clinical remission (S0)	14 (45.2)	10 (32.2)
Mild (S1)	16 (51.6)	15 (48.4)
Moderate (S2)	1 (3.2)	6 (19.4)
Severe (S3)	0 (0.0)	0 (0.0)
Endoscopic (Mayo) subscore, *n* (%)		
Mayo 0	6 (19.4)	13 (41.9)
Mayo 1	9 (29.0)	9 (29.0)
Mayo 2	13 (41.9)	5 (16.1)
Mayo 3	3 (9.7)	3 (9.7)
Endoscopic remission (Mayo 0/1)	15 (48.4)	22 (71.0)
Alternative 5-ASA formulation switched to		
MMX mesalazine	28 (90.3)	
Time-dependent, ethylcellulose coated[2]	2 (6.5)	
Sulfasalazine	1 (3.2)	
Median balsalazide dose (g, range)	5.3 (3.0-9.0)	-
Median equivalent mesalazine dose (g, range)[3]	2.1 (1.1-3.2)	3.6 (2.0-4.8)
Concurrent Medical therapy, *n* (%)		
Nil other	7 (22.6)	
Topical aminosalicylate	10 (32.2)	
Oral corticosteroid	1 (3.2)	
Azathioprine/mercaptopurine	14 (45.2)	
Methotrexate	3 (9.7)	
Anti-TNF biologic	0 (0.0)	
Other biologic	0 (0.0)	

[1]Median 3 mo after baseline–overall cohort data reported here (*i.e.*, either on alternative aminosalicylate or had resumed balsalazide).
[2]Marketed as Mezavant® (Shire Pty Ltd) and Pentasa® (Ferring Pty Ltd) in Australia respectively.
[3]Based on Balsalazide Product Information[7].

hospitalization or colectomy, as well as significant anxiety, psychological distress, and loss of work productivity. Hence, such a shortage poses a risk for not only the patient but health payers and the drug manufacturer.

The most striking finding of this study is perhaps the significant loss of balsalazide's market share resulting from the shortage to competitor 5-ASA formulations of approximately 40% which persisted even to 5 years in this cohort. Given the sudden, unexplained nature of the drug shortage for both clinicians and patients with no advanced notification of return of supply, all patients were switched immediately to alternative 5-ASA formulations in order to maintain treatment continuity. Once balsalazide was again available, it is perhaps unsurprising that given the loss of confidence in drug supply that most patients chose to remain on the alternative 5-ASA therapy. This study therefore presents a warning to drug manufacturers that despite a relatively short-duration, once-off drug shortage, the effects on patient and clinician confidence in a product may be far more lasting, especially where similarly effective, competing formulations are available. Indeed, a sustained loss of 40% of prior market share as depicted in this study highlights financial risks to pharmaceutical companies as a result of suboptimal manufacture

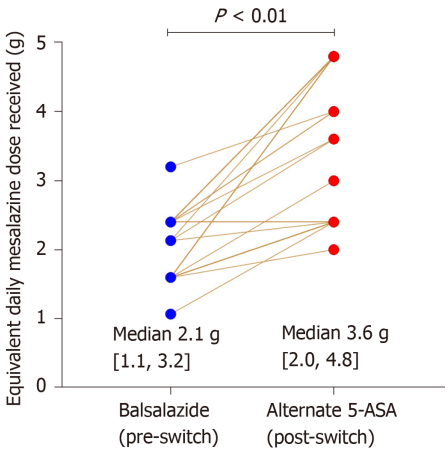

Figure 1 Change in equivalent mesalazine dose with switch from balsalazide to alternative aminosalicylate agent.

and supply-chain processes, especially in chronic diseases.

In this study there was no significant impact elicited in either clinical or endoscopic assessed disease activity on a per-group basis between those who remained on an alternative 5-ASA formulation post-switch and those who returned to balsalazide after drug supply returned, at each of the assessment timepoints to five years. Although this might imply the bioequivalence of the 5-ASA formulations, given the small sample size in this study no further conclusions can be made. It is important to reiterate that all switches were performed for non-medical reasons (*i.e.*, shortage) only and those switched for other reasons such as intolerance, active disease, or tablet burden were excluded from the study. However, on a per-patient basis, a greater proportion of patients suffered a symptomatic worsening than improvement of their colitis at initial review post-switch ($P < 0.01$) and there was a higher rate of flares requiring hospitalization through to five years (37% *vs* 0%, $P = 0.03$) despite no differences in rates of treatment escalation between the groups. One may therefore hypothesise that for a given individual, not all oral aminosalicylate preparations are equal and due to reasons including disparate tablet/granule composition, delivery system, pharmacodynamics and phenotypic differences, switching between agents within class may result in improved/worsened disease control and/or adverse effects. Regardless, these data exemplify the potential clinical sequelae of a drug shortage, which hitherto has not been well characterized in previous studies[13-15].

Notably, upon non-medical switching from balsalazide to an alternative 5-ASA formulation, approximately one-third of patients developed an adverse effect requiring drug cessation. This is a far higher proportion of adverse effects than typically seen in commencing 5-ASA agent/s in UC, and certainly higher than that reported in mesalazine registration trials[16,17]. Furthermore, many of the side effects appeared to be of idiosyncratic type (*e.g.*, abdominal pain, Figure 2), occurred rapidly within 1-2 wk of commencement and all resolved upon cessation and recommencing balsalazide. It is plausible therefore that at least a proportion of these adverse effects might be explained by a nocebo effect– *i.e.*, an effect occurring when negative expectations of the patient regarding a treatment cause the treatment to have a more negative effect than it otherwise would have, such as recently reported with the non-medical switching from originator to biosimilar infliximab in similar disease populations[18,19]. If so, this further illustrates the potential negative impact of a drug shortage on patients, especially in diseases like UC where psychological stress has been linked with flares and/or symptom provocation[20]. Another possible explanation is that, given the vast majority of patients were switched to MMX mesalazine, that this particular formulation might be the cause of adverse effects. Alternatively, the increase in side effects might have been explained by the almost universal increase in equivalent mesalazine dose received by patients switching to the alternative 5-ASA therapy (median increase of 1.6 g mesalazine), although multiple studies have demonstrated that adverse effects to mesalazine are not dose-dependent[21,22].

The authors acknowledge several limitations of this study, including the observational design and small sample size which limit the ability to make definitive conclusions given potential bias, or ascribe causality. However, this was an unselected, consecutive patient cohort who were all on the same treatment (balsalazide) prior to a non-medical therapeutic switch, were well phenotypically characterized (all mild-moderate UC), were all followed prospectively for five years

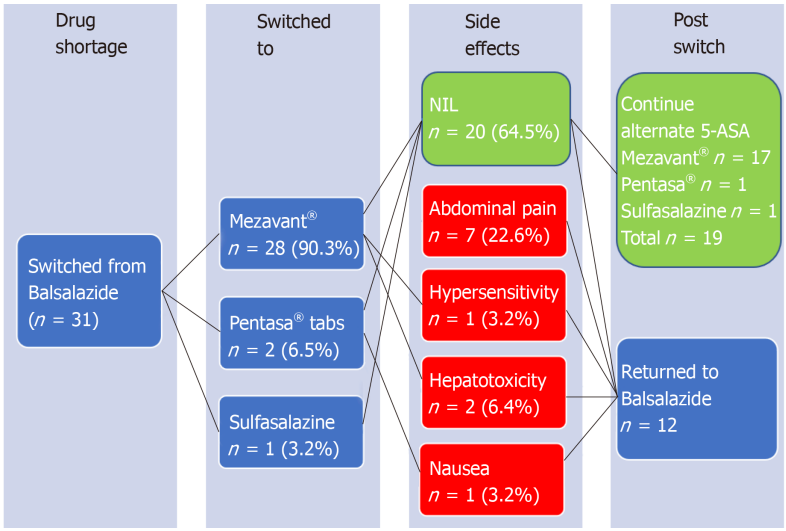

Figure 2 Flow chart depicting the switch to alternative aminosalicylate resulting from the balsalazide shortage, with adverse effect rate of 35.5% occurring immediately post-switch, which in all cases resolved upon return to balsalazide.

and all treatments and disease (including endoscopic) assessments were recorded, thus alleviating much of the potential bias. Moreover, the vast majority (over 90%) were switched to MMX mesalazine upon shortage of balsalazide, further aiding uniformity. Other limitations of the study included that endoscopic and clinical assessment of disease activity prior to and post-switch was not performed at strictly uniform timepoints and faecal calprotectin testing was not routinely available for most patients during this study. Assessment by CRP was performed at the clinic visits by these patients, but CRP has known poor sensitivity in the assessment of disease activity in UC[23]. Finally, concomitant medications were not controlled in keeping with the "real world" nature of this cohort, however it should be noted that none of the patients were on biologic therapy at the time of drug shortage though a significant proportion were on concurrent immunomodulators.

In summary, this study has demonstrated the deleterious effects of a drug shortage in ulcerative colitis, with a higher than expected proportion of patients exhibiting a worsening of disease and/or significant side effects upon substitution of their maintenance agent balsalazide with an alternative in the same class. Furthermore, this study demonstrated the adverse commercial impact of a drug shortage for the manufacturer, with a 40% loss of market share persisting even to five years post-shortage. Despite enhanced globalization, supply chains and technological advances, drug shortages are increasingly common and relative low incidence chronic diseases such as IBD appear at higher risk. Hence, this study highlights the threat posed by drug shortages to patients, clinicians, healthcare payers and pharmaceutical companies alike, and the need to explore ways to minimise such occurrences in future.

Table 2 Long term cumulative outcomes including rates of treatment escalation, colectomy and mortality in those who continued on alternative aminosalicylate therapy (*n* = 19) *vs* those who switched but then returned to balsalazide as soon as supply returned (*n* = 12), *n* (%)

Outcome (cumulative)	Post-switch review (baseline), *n* (%)		As of 3y follow-up[1], *n* (%)		As of 5y follow-up[1], *n* = (%)	
	Alternative 5-ASA[2]	Resumed alsalazide	Alternative 5-ASA[2]	Resumed balsalazide	Alternative 5-ASA[2]	Resumed balsalazide
Escalated to immunomodulator	14 (73.7)	5 (41.7)	16 (84.2)	5 (41.7)	16 (84.2)	5 (41.7)
Escalated to biologic	0 (0)	0 (0)	3 (15.8)	0 (0)	6 (31.6)	2 (16.7)
Hospitalised for flare[3]	0 (0)	0 (0)	3 (15.8)	0 (0)	7 (36.8)[a]	0 (0)[a]
Colectomy	0 (0)	0 (0)	1 (5.3)	0 (0)	1 (5.3)	0 (0)
All-cause mortality[4]	0 (0)	0 (0)	2 (10.5)	0 (0)	2 (10.5)	0 (0)

[1]Outcome occurring prior to or at timepoint.
[2]5-ASA: Aminosalicylae.
[3]First hospitalization counted for UC flare only.
[4]Both deaths in cohort were unrelated to ulcerative colitis (one due to sarcoma and one acute myocardial infarction).
[a]$P < 0.05$.

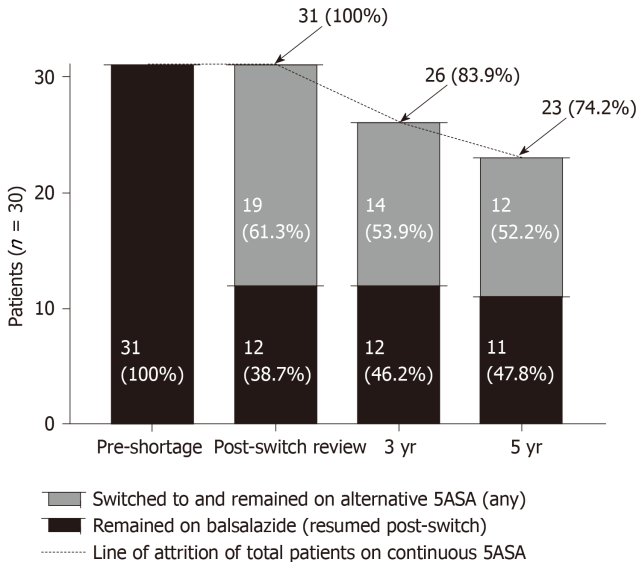

Figure 3 Long term outcome of balsalazide drug shortage on market share through to five years follow-up (compared to persistence on alternative aminosalicylate therapy).

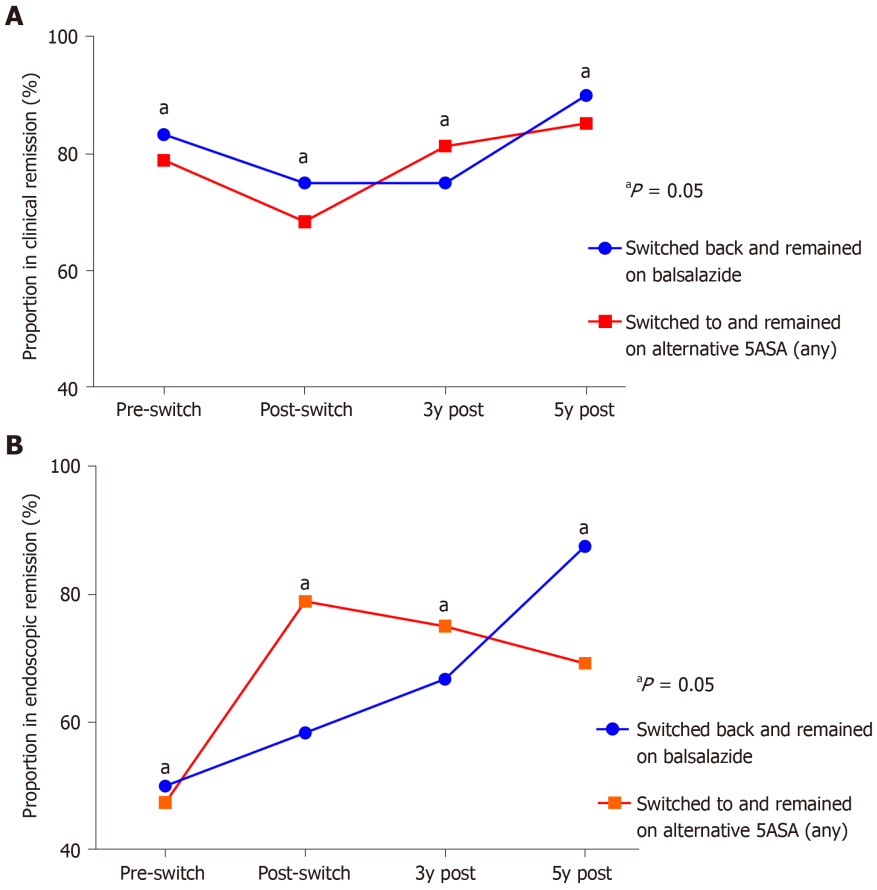

Figure 4 Comparison of rates of Clinical remission and Endoscopic remission over multiple timepoints in those who switched to and remained on alternative aminosalicylate *vs* those who switched back and remained on balsalazide. A: Clinical remission; B: Endoscopic remission.

ARTICLE HIGHLIGHTS

Research background

Drug shortages appear to be occurring more frequently, yet their clinical impact and sequelae are not yet well described. Here, a nationwide drug shortage of balsalazide occurred over several months in 2012-3, necessitating a sudden switch to alternative aminosalicyclate formulations.

Research motivation

In this study the impact of this balsalazide shortage was intensively assessed in a well characterized population of patients with ulcerative colitis over a five year period to assess short and long term effects of a drug shortage. We hypothesized that this and similar drug shortages can have significant detrimental impacts on disease course and patient outcomes.

Research objectives

This study aimed to elucidate the short and long term ramifications of a drug shortage in ulcerative colitis patients on (1) efficacy (including symptom-based, and objective disease assessments); (2) safety (including immediate adverse effects occurring after switching to alternative agents); and (3) the proportion of patients returning to the original product once supply resumed as a measure of loss of market share. This comprehensive, holistic assessment of drug shortage-related outcomes sets a benchmark for further quantitative research in this field.

Research methods

A prospective cohort study of patients on balsalazide for mild-moderate ulcerative colitis was conducted where, strictly due to the national shortage patients were switched to alternative 5-ASA and/or then returned to balsalazide once supply resumed. Clinical and disease activity assessments were performed at baseline pre-switch, then immediately and at 3 and 5 years after the drug shortage-imposed switch to assess short and long term sequelae.

Research results

Although in stable remission at the time of the drug shortage, almost half of the patients when switched from balsalazide had documented clinical worsening at their subsequent review, including several reporting side effects to the alternative formulation. Only a minority of patients returned to balsalazide after drug supply returned, equating to a loss of market share (within the same class) of approximately 40% even to five years post-shortage in this cohort.

These data highlight the importance of maintaining a seamless drug supply for both patients (given significant rates of disease worsening occurred, directly attributable to shortage), and drug manufacturers given the loss of market share engendered by even a short term drug shortage.

Research conclusions

In one of the first published studies of its kind to date, this study of a balsalazide shortage in UC patients exemplifies the detrimental impact of a drug shortage on long term patient, disease and commercial outcomes. Hence, patients, clinicians and drug manufacturers should be more aware and explore ways to address and minimize this growing problem worldwide.

Research perspectives

Further prospective, larger scale studies are needed to document the impacts of drug shortages in patients across multiple chronic and/or life-threatening diseases. By documenting the scope of this problem in this manner, hopefully long term solutions can then be instituted accordingly.

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REVIEW

Selective granulocyte and monocyte apheresis in inflammatory bowel disease: Its past, present and future

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Abstract

The etiology and pathogenesis of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, are not fully understood so far. Therefore, IBD still remains incurable despite the fact that significant progress has been achieved in recent years in its treatment with innovative medicine. About 20 years ago, selective granulocyte and monocyte apheresis (GMA) was invented in Japan and later approved by the Japanese health authority for IBD treatment. From then on this technique was extensively used for IBD patients in Japan and later in Europe. Clinical trials from Japan and European countries have verified the effectiveness and safety of GMA therapy in patients with IBD. In 2013, GMA therapy was approved by China State Food and Drug Administration for therapeutic use for the Chinese IBD patients. However, GMA therapy has not been extensively used in China, although a few clinical studies also showed that it was effective in clinical and endoscopic induction of remission in Chinese IBD patients with a high safety profile. This article reviews past history, present clinical application as well as the future prospective of GMA therapy for patients with IBD.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Granulocyte and monocyte apheresis; Therapy; Efficacy

Core tip: Conventional therapies for inflammatory bowel disease (IBD) patients including mesalazine, corticosteroids, and immunosuppressants have been used for decades with unsatisfactory outcomes due to ineffectiveness or side effects. Although the emerging biologic agents have revolutionized IBD treatment, severe opportunistic infections, primary or secondary loss of response, *etc*. are the major clinical concerns of clinicians and patients. In recent years, selective granulocyte and monocyte apheresis has been used in Japan, Europe, China and elsewhere for its advantages of satisfactory

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efficacy and high safety profile. Granulocyte and monocyte apheresis therapy is an important and promising therapeutic option for IBD patients.

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two chronic relapsing and remitting diseases, *i.e.* ulcerative colitis (UC) and Crohn's disease (CD). Millions of individuals worldwide are currently affected by this disease in terms of function and quality of life[1]. Although it is thought to be an immune disorder of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors[1-4], IBD is sometimes difficult to define due to either its history of unusual complexity or poor understanding of its etiology and pathogenesis. Therefore, IBD still remains incurable despite significant progress in recent years due to its treatment with innovative medicine. Clinical and experimental studies have indicated that the distressful flare-ups of IBD might be triggered by the impaired function of the intestinal barrier and a dysregulated immune response to the gut microbiota[5,6].

Treatment of IBD with aminosalicylates and corticosteroids has been used for decades with unsatisfactory outcomes due to either their poor effectiveness or side effects. In recent years, an array of emerging medical therapies including biologic agents, immune cell modulators, mucosal barrier enhancers and stem cells have revolutionized IBD treatment[7]. Nevertheless, therapies for IBD have been mostly empirical rather than based on understanding of the disease etiology. Furthermore, side effects of long-term medications are inevitable, even though medications are often initially effective in the majority of patients[8].

It is currently believed that an imbalance between pro-inflammatory and anti-inflammatory cytokines exist in patients with IBD, which is closely related to the onset and development of the disease[9,10]. Additionally, myeloid leukocytes, especially granulocytes and monocytes, have been shown to play an important role in the occurrence and development of IBD[11,12]. Based on this theory, selective granulocytes and monocytes apheresis (GMA) therapy targeting these cells and subsequently on inflammatory cytokines was invented and applied to IBD patients in Japan about 20 years ago. It has been generally accepted that GMA therapy is a non-pharmacological therapeutic option for IBD patients due to its good therapeutic effect and incomparable safety, especially when conventional therapies are ineffective[13,14]. This article reviews the past history, present application, and future prospect of GMA therapy for patients with IBD.

GMA HISTORY

The term "apheresis" is from the Greek "*apairesos*" or Roman "*aphairesis*" meaning to remove or take away something by force or a withdrawal. Before modern medicine, it was a common way to treat diseases, *e.g.*, by bloodletting. At that time, it was thought that the goal of treatment could be achieved by eliminating pathogenic factors from the blood[15]. The removal of plasma and return of red blood cells was first demonstrated in research animals at Johns Hopkins University in 1914. Since then, apheresis technology was gradually used in clinics, including centrifugation, plasmapheresis, plateletpheresis, photopheresis, *etc*. The technique of selective GMA originated from the separation of blood cells.

As early as the beginning of the 20th century, centrifugation was used to selectively remove blood components that were thought to activate or promote the occurrence of diseases to achieve a therapeutic goal through non-drug therapy. Separation of blood cells was first used for leukemia, tumors, rheumatoid arthritis and other diseases and later gradually applied to treat IBD[16]. However, centrifugation has certain disadvantages of being expensive to perform and difficult to handle.

In the 1980s, a novel extracorporeal leukocyte removal filter with a commercial

name of Cellsorba was developed by Asahi Medical Co. Japan. The filter consists of non-woven polyester cylindrical fabric. Using this filter, one could partially remove leukocytes from the whole blood during extracorporeal circulation, and it was approved for therapeutic use by the Japanese government in 1989. Cellsorba is simpler to operate and more efficient in the removal of granulocytes and monocytes and hence has greater clinical efficacy than centrifugation[16]. It has been found that the Cellsorba column is capable of removing about 1.6×10^{10} white blood cells per session including almost 100% of neutrophils and monocytes and 30%-60% of lymphocytes.

Studies have shown that the leukocyte removal filter could reduce the number of activated leukocytes as well as serum levels of pro-inflammatory cytokines[17-19]. Selective GMA, also known as granulocytapheresis, could selectively remove granulocytes and monocytes using a device commercially named Adacolumn, which was developed by the Japan Immunoresearch Laboratories Co., Ltd. of Takasaki, Japan. The column (G-1 granulocyte removal column) is packed with cellulose acetate beads. About 65% of granulocytes, 55% of monocytes and a significant fraction of lymphocytes are removed from peripheral blood passing through the column[20]. In the early 2000s, Japan national health reimbursement scheme introduced GMA as an induction of remission therapy for UC patients[21]. In the same year, Adacolumn was approved by the Ministry of Health of Japan for treating UC patients. Afterwards, Adacolumn became available for clinical application for IBD patients in the European Union countries after it was CE marked[22]. From then on, there have been accumulating reports on clinical efficacy and safety of Adacolumn in patients with IBD from Japan as well as from European countries[23-28]. In 2013, GMA therapy was approved for Chinese IBD patients by the Government Health Authority, and since then it has been applied in clinics to treat IBD patients in China mainland[29].

GMA CURRENTLY

GMA equipment

The Adacolumn (G-1 column), 206 mm in length, 60 mm in diameter and 335 mL in capacity, is made of polycarbonate and filled with 220 g of cellulose acetate beads of 2 mm in diameter (adsorptive carriers) bathed in 130 mL sterile saline[30] (Figure 1)[31]. The Adacolumn apheresis system consists of four components: The column, the blood circuit lines, Adamonitor and the pump. The column and its blood circuit lines are allowed for single use. The Adamonitor is the center of the system and is formed by a blood pump and four other functional units[20,22]. The pump of the Adacolumn system has special functional units, including a flow rate and time setting panel, a pressure monitor as well as a fault detecting alarm system. With the help of these functional units, if the actual pressure of the apheresis blood does not match the preset values, then the system will alarm and automatically stop working. Likewise, in the event of other abnormal conditions, the system will recognize it and alarm. Sometimes it will automatically switch off to ensure the safety of apheresis procedures[22].

Mechanisms of GMA

GMA reduces inflammatory leukocytes and inhibits their infiltration: Elevation in number and activity of neutrophils and monocytes in peripheral blood contributes to the basic pathophysiology of IBD. In patients with IBD, peripheral circulating activated granulocytes, monocytes and macrophages are increased in number and subsequently lead to an increased level of circulating pro-inflammatory cytokines and infiltration of intestinal mucosa by these inflammatory cells, which are significantly correlated with intestinal inflammation level[11]. Therefore, removal of these inflammatory cells should be theoretically beneficial to patients with IBD[9].

Cellulose acetate beads inside the Adacolumn are capable of selectively adsorbing circulating neutrophils and monocytes by binding to IgG fragments (Fcγ) and immune complement complexes[32], which serve as a "connecting bridge" between leukocytes and the beads (Figure 2). A significant reduction of CD14(+) CD16(+) monocytes in peripheral blood of GMA-treated IBD patients can be observed[33]. One study showed that soluble cell adhesion molecule-1 and soluble vascular cell adhesion molecule-1 were significantly increased in the peripheral blood of patients with IBD and closely related to the degree of tissue inflammation[34]. An *in vitro* study observed that the concentration of soluble cell adhesion molecule-1 and soluble vascular cell adhesion molecule-1 in blood samples was significantly decreased after incubation with acetate beads as adsorption carrier at different temperatures when compared with that in the control group without acetate beads incubation[35]. Therefore, GMA is also capable of inhibiting leukocyte migration by downregulating expression of leukocyte related adhesion molecules and thereby affecting the adhesion between

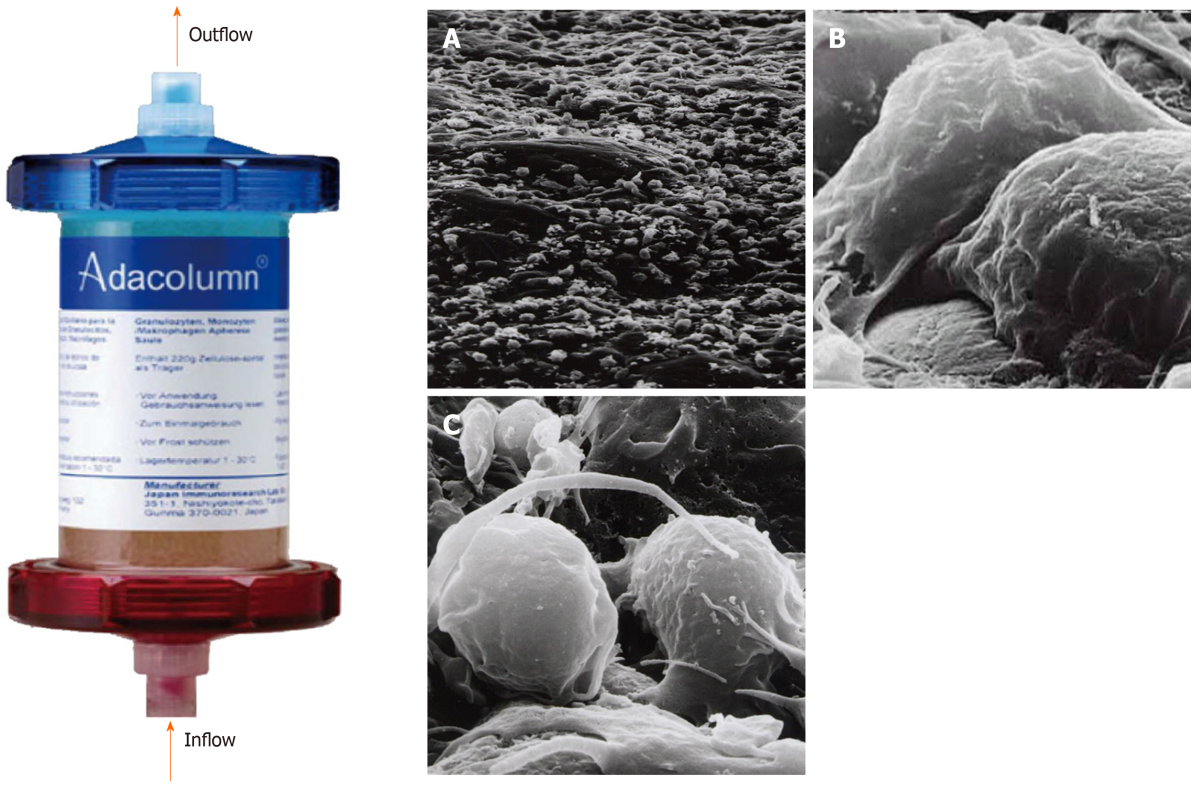

Figure 1 Photograph of Adacolumn and scanning electron photomicrograph of the acetate beads after treatment. Adacolumn is filled with cellulose acetate beads of 2 mm in diameter (adsorptive carriers) bathed in sterile saline. The blood from the antecubital vein of one arm flows into the column and returns to the antecubital vein in the contralateral arm. A: A low power view (400 ×) of the acetate beads in a column after treatment with cells covering the surface of the carrier; B: Viewed at 10000 ×. Neutrophils were adsorbed onto the beads; C: Viewed at 12000 ×. Activated monocyte/macrophages are seen (taken by Dr. A. Saniabadi of Japan Immunoresearch Laboratories). Modified from reference[31].

cells and vascular endothelium at the initial stage. One study indicated that CX3CR1 played an important role in regulating the dynamic balance of intestinal macrophages, bacterial translocation and inflammatory effector Th17 response in patients with IBD[36]. Pro-inflammatory monocytes are able to highly express α4 integrin and CX3CR1 in patients with active UC, while GMA is able to selectively remove CD14+CD16+CX3CR1+ monocytes and increase CD14hiCD16-CCR2low "immature" monocytes and consequently inhibiting the adhesion and chemotaxis of pro-inflammatory monocytes to a certain extent[37].

GMA affects functions of other immune cells: One study observed that granulocytes removable by the Adacolumn from peripheral blood were mostly CD10-positive granulocytes, but the number of myeloid CD10-negative premature granulocytes with low pro-inflammatory function increased significantly after GMA therapy. This indicates that GMA therapy may play its therapeutic role indirectly by promoting the migration of "less-inflamed" premature granulocytes from bone marrow to peripheral blood making the granulocyte level in the peripheral blood unchanged[38]. Another study showed that the Adacolumn adsorption column was involved in the increased induction of myeloid-derived suppressor cells, which are strong anti-inflammatory cells, thus regulating the inflammatory response through immune cells to relieve the disease[39]. $CD4^+CD25^+Foxp3^+T_{reg}$ cells are necessary for the maintenance of autoimmune tolerance. Kamikozuru *et al*[40] showed that after five sessions of GMA in active UC patients who achieved remission, the number of $CD4^+CD25^+Foxp3^+T_{reg}$ in the peripheral blood increased to the level of the normal control group at the 10^{th} wk. A clinical study by Muratov *et al*[41] showed that $CD4^+$ T cells producing IFN-γ in peripheral blood of active IBD patients were significantly reduced after GMA therapy, and Waitz *et al*[42] found that the number of myeloid dendritic cells in the peripheral blood of patients with active UC was significantly higher than that of the normal control group. However, the number of myeloid dendritic cells in peripheral blood after GMA therapy was significantly lower. The mechanism may be that dendritic cells can express a variety of receptors, including Fc gamma, which can be absorbed by the cellulose acetate beads of the Adacolumn, resulting in a transient decline of dendritic cells in the peripheral blood and increase intestinal tolerance to different antigens.

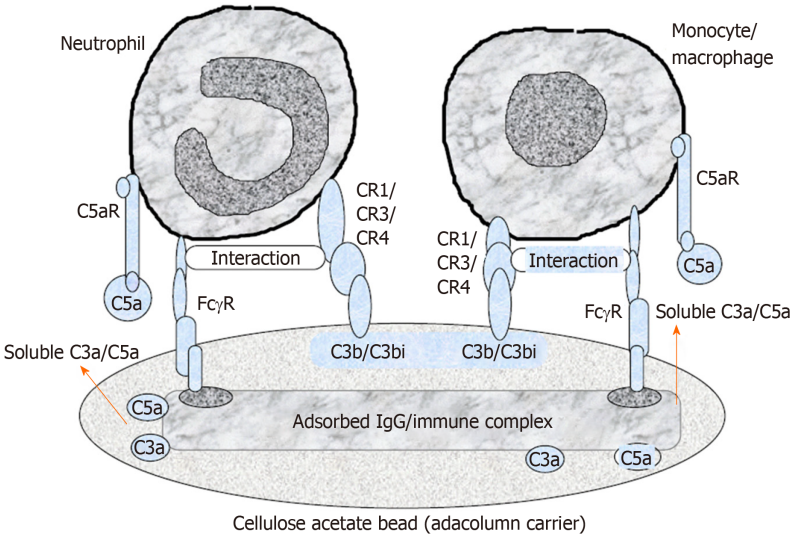

Figure 2 A schematic diagram of the selective adhesion of myeloid granulocyte and monocyte to cellulose acetate carriers. Cellulose acetate beads inside the Adacolumn are capable of selectively adsorbing circulating neutrophils and monocytes by binding to IgG fragments (Fcγ) and immune complements complexes. Lymphocytes are not absorbed as they rarely express complement receptor. Modified from reference[31].

GMA regulates pro-inflammatory and anti-inflammatory cytokines: As a pro-inflammatory cytokine, TNF-α plays a very important role in IBD. Its level in active IBD patients is significantly higher than that in normal subjects. One study showed that GMA therapy could significantly decrease peripheral blood levels of TNF-α, IL-1β, IL-6 and IL-8 in active IBD patients to alleviate the disease[38], and the intestinal tissue levels of these cytokines were significantly lowered in IBD patients who had achieved clinical or endoscopic remission but remained unchanged in patients without remission after GMA therapy[43]. It is known that TGF-β1 is a kind of pleiotropic cytokine as well as the most powerful intestinal immunosuppressant. Cellulose acetate in the Adacolumn can absorb soluble human leukocyte antigen I in peripheral blood, and soluble human leukocyte antigen I induces the production and release of soluble Fas ligand, which further leads to the production of TGF-β1[44,45], hence inducing the immune suppression effect. On one hand, IL-1, including IL-1α and IL-1β, is the major pro-inflammatory cytokine in the early inflammatory response and is significantly enhanced in its expression in the inflamed intestinal tissues, which is correlated well with the disease activity. On the other hand, IL-1Ra, a strong anti-inflammatory inhibitor of IL-1, is increased in active UC patients who responded well to GMA therapy, but no significant change is seen in IL-1Ra levels in patients who did not respond to GMA therapy[46]. This means that balance of IL-1Ra/IL-1 is very important in regulating inflammatory response in IBD patients.

GMA for IBD

GMA for UC: The first clinical trial of GMA for UC was reported in 2001. It was a multicenter controlled study with a total of 53 patients with active UC receiving five sessions in consecutive weeks of GMA therapy in combination with prednisolone at 14 hospitals in Japan[21]. By the 7th wk, 58.5% of the patients had achieved remission or improved, and prednisolone dosage was gradually reduced. Only eight non-severe adverse events in 5 patients were reported. Therefore, the first clinical trial indicated that GMA was a potentially effective and safe way to induce remission as well as tapering steroid dosage[21]. Since then, a large number of clinical applications and studies have been conducted in Japan, most of which showed satisfactory clinical and endoscopic outcomes and proved that GMA is an effective and safe way for UC patients who experienced tapering of corticosteroids as well as lowering the colon resection rate[24,47-54]. A significant study was reported in 2009 by Hibi *et al*[55] in which 656 severe or refractory UC patients in 53 medical institutions were observed over 7 years. The results showed the clinical response rates of severe, moderate and mild patients were 63.2%, 65.7% and 80.4%, respectively. Patients treated with GMA had a lower clinical recurrence rate and longer sustained remission.

In recent years, biologic agents and immunosuppressants have been increasingly used in IBD. However, some IBD patients responded poorly to these agents. Many clinical trials were conducted to observe the effect of GMA for IBD patients who failed or were refractory to pharmacological therapy. D'Ovidio *et al*[24] treated 12 mild to

moderate steroid-dependent/refractory UC patients by GMA. After 6 wk, mucosal healing was accomplished in 3 patients, partial mucosal healing in 8 patients, and 1 patient had no response. In 2016, in a single-arm, open-label, multicenter trial[56] conducted in 18 centers in the United Kingdom, France and Germany, 84 moderate-to-severe active UC patients having poor response or intolerance to immunosuppressant and/or biologics were enrolled. Each patient received GMA therapy of one session per week with the Adacolumn. After continuous apheresis for 12 wk, 33 of the 84 patients achieved clinical remission, and 47 achieved a clinical response. For patients with previous immunosuppressant and/or biologic failure, the clinical remission rate was 30.0%. Similar results were also found from other reports[50,57]. These studies indicated that the Adacolumn apheresis is effective in induction of remission in patients with steroid-dependent UC who failed immunosuppressant and/or biological agents.

Apart from these clinical trials aiming at induction of remission, the effectiveness of maintenance in UC patients treated with GMA was also observed. In a multicenter study at 24 medical institutions in Italy[58], a total of 230 patients (including 194 UC patients) received at least one session of GMA therapy and were then followed up for 1 year. The results showed that 77.7% of UC patients obtained positive outcomes at 3 mo and 87.1% at 12 mo. Similar results were also observed in other studies[27,41]. Furthermore, it seems to be equally effective in relapsed patients who have achieved remission by previous GMA therapy[59].

In 2011, a retrospective, observational, multicenter study was conducted for cytomegalovirus (CMV) positive UC patients[60]. In this study, CMV-positive UC patients were treated with either additional GMA (11 patients) or immunosuppressant (9 patients) after ineffective antiviral treatment. In the GMA group, 9 patients achieved remission and 2 underwent colectomy. In the immunosuppressant group, 4 achieved remission but 5 underwent colectomy. Therefore, it was concluded from this study that GMA was more effective in UC patients with opportunistic CMV infection as compared with conventional drugs like immunosuppressants. Nevertheless, additional studies for GMA therapy for CMV-positive IBD patients need be performed in the future because a conclusion should be reached with care from this retrospective study with a small sample size. However, this study demonstrated that GMA was safer than immunosuppressants in patients with opportunistic infections.

Relapse is an unlucky clinical feature of patients with UC. An open-label, prospective, randomized, controlled study[61] from the United Kingdom was conducted aiming at prevention of relapse in UC patients by GMA therapy. Sixty UC patients in remission but with fecal calprotectin over 250 mg/g (at high risk of clinical relapse) were enrolled. Twenty-nine patients received five sessions of weekly GMA, and thirty-one patients were kept on maintenance therapy. After 6 mo follow-up, 72.4% of the GMA-treated patients were still in remission, while only 32.3% in the control group were still in remission. Therefore, it was concluded from the studies above that selective leukocytapheresis significantly reduces recurrence rates and delays the time to relapse. Furthermore, GMA in combination with biologics also yielded satisfactory clinical outcomes. In a retrospective study reported by Tanida *et al*[62] in 2018, nine refractory UC patients received combination therapy with adalimumab plus intensive GMA. Over half of the nine patients displayed clinical remission at 10 wk, and 33.3% displayed remission at 52 wk under subsequent maintenance monotherapy of adalimumab.

Although GMA therapy has been proven effective and safe in patients with UC, there was a report with contradictory results. In a randomized, prospective, double-blind, sham-controlled trial[63] conducted at 36 centers in the United States and Canada, 168 patients with moderate to severe active UC were enrolled and assigned randomly to either GMA group (84 patients) or sham-treatment group (84 patients). The results showed that the clinical remission and response rate in the GMA group were 17% and 44%, respectively, while the clinical remission and response rate in the sham-treatment group were 11% and 39%, respectively. No differences between the two groups were found indicating that GMA was unsatisfactory in treating patients with moderate to severe UC. The conclusion from this study contradicts that from the majority of other studies. It is known that the best responders to GMA therapy are UC patients of short disease duration with no previous medications and steroid naïve UC patients[53,64]. So the possible explanation for this contradiction may be that patients enrolled in this trial did not fall into the category of best responders.

In summary, GMA is an effective and safe therapeutic option for moderate to severe UC patients, particularly for those who are refractory to or dependent on corticosteroids, which can be tapered or avoided. At present, no one can conclude that GMA can be used as a first-line therapy, but at least as an alternative choice for patients with UC (Figures 3 and 4).

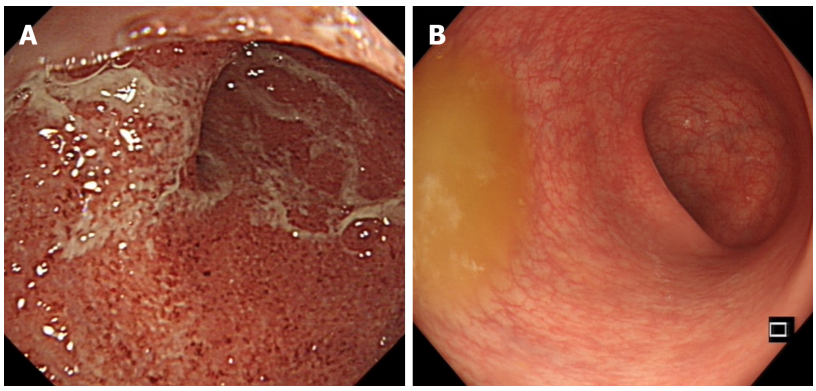

Figure 3 Endoscopic photographs of an ulcerative colitis patient who responded well to selective granulocyte and monocyte apheresis. A: Endoscopic photograph before granulocytes and monocytes apheresis therapy; B: Endoscopic photograph after ten sessions of granulocytes and monocytes apheresis therapy.

GMA for CD: The efficacy of GMA in patients with CD was first reported by Matsui *et al*[65], who treated 7 CD patients refractory to conventional therapy. The patients received five or six sessions of weekly GMA in combination with the previous conventional therapy. The results showed that 5 patients achieved clinical remission while the other 2 patients did not respond. In another clinical study that enrolled 21 active CD patients, all of the patients achieved significant improvement 7 wk after weekly GMA for 5 consecutive weeks as an adjunct to conventional therapy evaluated by CDAI, IOIBD and IBDQ scores[66]. Apart from the studies showing the usefulness of GMA, other clinical studies including case reports also verified the effectiveness of GMA in patients with CD[67-70]. In 2018, Tanida *et al*[71] reported three patients with refractory CD treated with intensive GMA plus ustekinumab. At wk 10, clinical remission was achieved for all the patients. Therefore, at present it is believed that GMA or in combination with biologics can be used to induce remission in CD patients.

As for maintenance of remission, a clinical case report from Spain showed that one steroid-dependent CD patient experienced no clinical and endoscopic relapse for 12 mo with the combination of infliximab and GMA therapy[25]. Another CD patient with severe fistula refractory to conventional therapy also achieved sustained remission by GMA therapy[28]. However, there were a number of CD patients who obtained disappointing outcomes with GMA therapy. In 2013, a randomized, double-blind, sham-controlled study was reported by Sands *et al*[72]. They enrolled 235 patients with moderate to severe active CD from the United States and Canada, and all the patients finished ten sessions of GMA therapy. The results showed that clinical remission and response rate in the GMA group was 17.8% and 28.0%, respectively, while the clinical remission and response rate in the sham-treatment group was 19.2% and 26.9%, respectively. In another clinical study involving 12 patients with steroid dependent CD who received weekly GMA therapy, only 1 patient experienced no relapse within 6 mo of follow-up in spite of the initial clinical remission in 70% of the patients[57]. From the above data, it seems that the effectiveness of GMA in CD patients is not as good as in UC as illustrated by a meta-analysis, which concluded that GMA therapy in UC demonstrated a significant higher clinical efficacy than CD[13]. The possible reasons for the difference of effectiveness of GMA therapy between UC and CD await explanation. One possible reason may lie perhaps in the different intestinal neutrophil infiltration between the small intestine and the colon[14].

Optimizing GMA for IBD: Based on clinical trials, the efficacy rate was as high as 100% for initial UC patients and over 80% for steroid naïve patients[52,53]. The best responders to GMA therapy are UC patients with short disease duration and no previous medications and steroid naïve UC patients[32,52,53,73]. In non-responders, deep colonic lesions or loss of extensive mucosal tissues are usually observed by endoscopic examinations[52,64]. GMA is a time dependent therapy for IBD patients; several weeks may be needed before achieving favorable clinical outcomes. In addition, five sessions of GMA therapy are generally good for patients with a short course of the disease, while patients with recurrent episodes, especially steroid dependent or refractory, usually require ten sessions to achieve remission[32,49,56].

Based on its frequency of sessions, GMA therapy is classified into two therapeutic protocols: Regular and intensive GMA. In regular GMA, one session per week is carried out for five to ten sessions, whereas in intensive GMA, two sessions per week

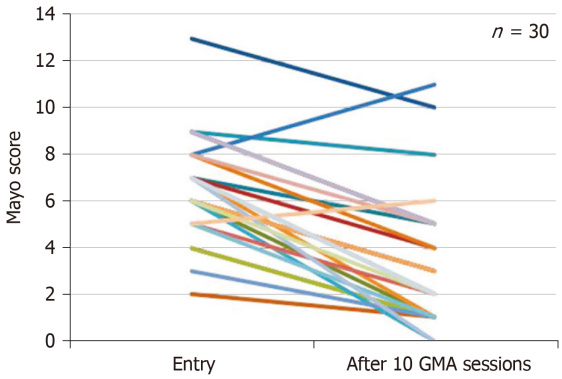

Figure 4 Changes of Mayo scores in 30 ulcerative colitis patients at entry and after ten granulocytes and monocytes apheresis sessions. Mayo scores were significantly decreased after ten granulocytes and monocytes apheresis sessions compared with that at entry[29]. GMA: Granulocytes and monocytes apheresis.

are required for a total of five to ten sessions[52,55]. Sakuraba *et al*[74] reported a prospective multicenter study involving 112 UC patients who were divided into two groups: Regular GMA group and intensive GMA group. The results showed that intensive GMA therapy has a significantly higher remission rate than regular GMA therapy. Treatment duration and volume of blood infusion in the Adacolumn of a single GMA session may also influence the effectiveness of GMA as seen in the study by Kanke *et al*[51]. They showed that 90 min for each GMA session has a significant better outcome as compared to routine 60 min of treatment. Yoshimura *et al*[75] attempted to increase the blood volume perfusion from 1800 mL to over 3000 mL per GMA session, which seemed to have yielded a significantly better clinical outcome with no safety concerns.

GMA for special IBD patients

GMA for children and adolescent IBD patients: IBD is featured by its high morbidity in children and adolescents in whom growth and development may be affected by the disease and by the supposed life-long pharmacological treatment as well. Therefore, non-pharmacological treatment appears to bear significant importance for children and adolescent IBD patients[76].

In 2003, the first clinical report of GMA therapy for pediatric IBD patients was published by Tomomasa *et al*[77]. They treated 12 steroid-refractory IBD children with an average age of 12 years using GMA therapy (five to ten sessions). Nine of the twelve patients achieved clinical remission, and two patients had no response. The dosage of steroid was tapered in all patients. Four of the nine patients relapsed in an average of 3.5 mo after the last GMA session. The other patients remained in remission until an average of 22.8 mo. Similar results had also been reported by Ruuska *et al*[78]. In a single center trial[79], a total of 24 children and adolescents with IBD who failed mesalazine or sulphasalazine were enrolled. After GMA therapy in combination with prednisolone, all the patients obtained remission. Furthermore, in a clinical trial[80] involving 53 pediatric/adolescent patients, the incidence of adverse events was 18.9%, a figure, however, higher than that in all 437 patients (11.4%).

From most of the above studies, one can see that GMA is effective and well tolerated in children and adolescent IBD patients who have failed conventional drug therapy, and GMA in combination corticosteroids yielded better clinical outcomes. However, there have been no sufficient clinical data to verify the effectiveness of GMA therapy in children and adolescents IBD patients, so more clinical studies are needed to address this question.

GMA for pregnant IBD patients: Clinical and epidemiological studies have shown that the fertility period was usually at the peak incidence of IBD patients, and the disease itself is an important risk factor for pregnancy. The fertility rate of IBD patients is significantly lower than that of healthy people due to disease activity, nutritional status, surgery and drug treatment. Therefore, it is challenging to manage pregnant IBD patients[81,82]. Theoretically speaking, GMA therapy could be safe and effective for pregnant IBD patients. However, at present most of the published studies were case reports. In 2006, Okada *et al*[83] reported a 30-year-old pregnant woman of 13 wk gestation with severe steroid-dependent UC who was treated by Cellsorba leukocytapheresis. The patient successfully achieved a rapid improvement after the first GMA session, and clinical remission was obtained 2 wk later. The patient delivered a full-term healthy baby during the remission stage. Another three case

reports involving 5 pregnant UC patients showed satisfactory responses to GMA therapy with smooth delivery and an absence of adverse events[84-86].

Although evidence of effectiveness of GMA in pregnant IBD patients are based upon case reports, it might become the first-line therapy for pregnant IBD patients due to its safety. Of course, more research is needed before GMA therapy is generally accepted as the first-line therapy for pregnant IBD patients.

Safety of GMA

As a non-pharmacological therapy, GMA is incomparable to other therapies regarding its safety[87]. The largest clinical study to date was performed by Hibi *et al*[55] who followed 656 UC patients treated by GMA from 53 centers in Japan starting in 2009 for 7 years. The results showed that GMA therapy had a very high safety profile with only mild or moderate adverse events related to GMA. In a multicenter study[21], a total of 53 patients were treated with GMA therapy in combination with prednisone for 5 wk. Only eight mild adverse events were observed in 5 patients, and no patients ceased the treatment due to adverse events. In another study, no significant differences regarding safety were found between patients receiving five and ten GMA sessions as reported by Dignass *et al*[88] who divided 196 patients with moderate to severe steroid-dependent or steroid-refractory UC into two groups of five and ten GMA sessions.

In a meta-analysis report involving nine randomized trials of GMA therapy[13,89], the most common adverse events were headache and flushing, and patients treated with GMA had a significant lower incidence of side effects than conventional therapies, *i.e*. corticosteroids. Besides, no serious adverse reactions had been reported in the children and pregnant women IBD patients who received GMA therapy[78,84,90].

The use of anticoagulant is indispensable for GMA therapy. Sawada *et al*[91] analyzed 832 patients from 116 medical facilities in Japan for safety of anticoagulant use of nafamostat mesylate or heparin. The main side effects in patients using nafamostat mesylate were mild headache (2.2%), nausea (1.3%) and fever (0.9%), while in patient using heparin, the main side effects were decreased platelet count (2.7%), nasal congestion (1.8%) and pain in the vascular access sites (1.8%). Apart from these mild side effects, no serious adverse events were observed in patients using either nafamostat mesylate or heparin. In summary, adverse events of GMA therapy are rare, mild and well tolerated.

FUTURE OF GMA

As a non-pharmacological therapy, GMA has been demonstrated to be effective and safe for patients with IBD. Nevertheless, most of the clinical trials and literature of GMA therapy came from Japan. It has not been used extensively on a global scale, particularly in China, although it was approved by China State Food and Drug Administration for IBD patients in 2013. Furthermore, its effectiveness in IBD patients, particularly in CD patients, was doubted by some authors especially in a biological era when many biologic agents and immunosuppressants have been extensively used for IBD. Besides, price is also one of the main factors limiting the clinical usage of GMA therapy when cost-effectiveness perplexes both doctors and patients. Therefore, much needs to be done before it can be accepted as a therapeutic option for IBD patients worldwide.

Mechanisms of GMA

GMA targets inflammatory immune cells such as granulocytes and monocytes /macrophages to alleviate intestinal inflammation in IBD patients[14]. In addition, other immune cells such as T cells, B cells and dendritic cells are also involved in the pathogenesis of IBD[40,42]. At present, although there have been a few studies on the influence of GMA on these immune cells[40-42], it is unknow how they contribute to the clinical effectiveness of GMA in patients with IBD. It is known that gut microbiota play an important role in the pathogenesis of IBD[6,92], but research must be done to determine whether GMA therapy influences the gut microbiota. One study (our unpublished data) has shown that the unfavorable gut microbiota could be improved by GMA therapy in patients with UC. It is possible that GMA therapy could exert its therapeutic effect by improving the steady state of gut microbiota in IBD patients. However, more studies are needed before we are able to answer these questions.

Re-evaluation of GMA: Many reports have verified the effectiveness of GMA for induction of remission in IBD especially in UC patients. However, most of them were case reports or clinical trials of small size from Japan and Europe. Therefore, more large-scale, multicenter prospective studies are needed to further verify the

effectiveness of GMA therapy for IBD. Furthermore, the effectiveness of GMA therapy in CD patients is controversial in limited clinical trials to date. Although GMA therapy may be effective for induction of remission in patients with relapsed UC, future studies should be focused on its effectiveness as a maintenance therapy[76,93]. Besides, it is also worth looking for predictive factors for responders to GMA therapy.

GMA for special IBD patients

The effectiveness of GMA has been verified in children and adolescent IBD patients[94]. In theory, GMA can be used in IBD patients of any age, but there are limited clinical trials of GMA in children and adolescent IBD patients. Due to the differences in disease characteristics, body weight and circulatory status between children and adults, future studies are needed to determine the appropriate candidates as well as safety of GMA therapy for children and adolescent IBD patients. Fetal safety in pregnant patients with IBD who receive pharmacological treatment during pregnancy has always been a common concern of both doctors and patients. Although most of the drugs for IBD patients are at relatively low risk to fetal safety, there are still insufficient data to prove that various drugs are safe for patients with IBD in pregnancy in terms of miscarriage or malformation. Therefore, the choice of drugs for patients with IBD during pregnancy is difficult for both doctors and patients[83,84]. Because it is a non-pharmacological technique in which no any medications are involved during the procedures, GMA is much safer for IBD patients in pregnancy as evidenced by several clinical reports[83-85]. However, future clinical trials and observation of large-scale trials are needed before GMA becomes accepted as a safe and effective therapy for IBD patients during pregnancy.

GMA for other autoimmune diseases

GMA therapy was invented by Japanese scholars for patients with IBD. Due to its effectiveness and safety, especially its effect on anti-inflammatory cytokines, GMA therapy has also been used to treat other autoimmune diseases. In Italy, Morabito *et al*[95] treated nine patients with alcoholic hepatitis with GMA. The results showed that GMA therapy could reduce circulating inflammatory markers and improve the patient's clinical status. In addition, GMA also has therapeutic effects on patients with Bechet's disease[96] and rheumatoid arthritis[97]. With more clinical applications, it is hopeful that GMA therapy could be used clinically for other autoimmune diseases apart from IBD.

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MINIREVIEWS

Non-alcoholic fatty liver disease and Atherosclerosis at a crossroad: The overlap of a theory of change and bioinformatics

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Abstract

Atherosclerosis (ATH) and non-alcoholic fatty liver disease (NAFLD) are medical conditions that straddle a communal epidemiology, underlying mechanism and a clinical syndrome that has protean manifestations, touching every organ in the body. These twin partners, ATH and NAFLD, are seemingly straightforward and relatively simple topics when considered alone, but their interdependence calls for more thought. The study of the mutual relationship of NAFLD and ATH should involve big data analytics approaches, given that they encompass a constellation of diseases and are related to several recognized risk factors and health determinants and calls to an explicit theory of change, to justify intervention. Research studies on the "association between aortic stiffness and liver steatosis in morbidly obese patients", published recently, sparsely hypothesize new mechanisms of disease, claiming the "long shadow of NAFLD" as a risk factor, if not as a causative factor of arterial stiffness and ATH. This statement is probably overreaching the argument and harmful for the scientific credence of this area of medicine. Despite the verification that NAFLD and cardiovascular disease are strongly interrelated, current evidence is that NAFLD may be a useful indicator for flagging early arteriosclerosis, and not a likely causative factor. Greater sustainable contribution by precision medicine tools, by validated bioinformatics approaches, is needed for substantiating conjectures, assumptions and inferences related to the management of big data and addressed to intervention for behavioral changes within an explicit theory of change.

Key words: Non-alcoholic fatty liver disease; Fatty liver; Arterial stiffness; Bioinformatics; Methodology of research

Core tip: Atherosclerosis and non-alcoholic fatty liver disease straddle a communal epidemiology, underlying mechanism and a clinical syndrome with protean manifestations, touching every organ in the body. Current therapeutic evidence supports

the recommendation of addressing changes toward healthier lifestyles, including diet and physical exercise, in atherosclerosis and non-alcoholic fatty liver disease, even when defined only by non-invasive methodology. Pathway-based analysis are elucidating key molecular mechanisms underlying complex diseases addressing the joint effect and integrality as function unit of multiple genes, exploring large-scale "-omics" data. No element suggests, apart from naïve statistics, that one condition affects the other directly by any mechanism.

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INTRODUCTION

According to World Health Organization, ICD-11 for Mortality and Morbidity Statistics (Version: 04/2019), non-alcoholic fatty liver disease (NAFLD) is characterized by fatty liver related to insulin resistance in the absence of significant alcohol consumption. It embraces a pathological spectrum from simple steatosis to steatohepatitis. 10%-20% have steatohepatitis (non-alcoholic steatohepatitis), which can progress to cirrhosis and hepatocellular carcinoma[1]. NAFLD and non-alcoholic steatohepatitis are increasingly a cause of cirrhosis and hepatocellular carcinoma globally. This burden is expected to increase as epidemics of obesity, diabetes and metabolic syndrome continue to grow[2]. Atherosclerosis (ATH) is a chronic disease of the arterial wall, and a leading cause of death and loss of productive life years worldwide. Its distinctive feature is a hardening of any artery specifically due to atheromatous plaques[3].

These conditions are increasingly recognized in primary care and specialist practice, which is largely due to the greater availability of non-invasive diagnostic tools. Nonetheless, quantifying future disease burden has always been challenging due to paucity of data in important areas, mandating an international, concerted effort to improve our understanding[4].

ATH and NAFLD are medical conditions that straddle a communal epidemiology, underlying mechanism and a clinical syndrome that has protean manifestations, touching every organ in the body[5]. Both entities stem from pathology, which seems certain and unarguable when considered alone, but when considered together, areas of uncertainty arise[6]. Interrelationship between the two conditions are many, sharing epidemiology and risk factors[7] which call for behavioral change interventions both in prevention and in therapy, but also challenging multi-factorial mechanisms by the quest of omics approaches[8], requiring computational biology analysis[9]. The conceptual milieu implies the overlap of a realistic theory of change, because implies lifestyle interventions focused on risk factors, with bioinformatics tools for the management of big data not comprehensive of all recognized risk components and indexes[10].

In the real clinical world, easy-to-assess proxies of ATH are frequently preferred: It is needed to have the awareness of using them as assumptions more than as well supported reference standards[11]. Accordingly, the present overview focuses to briefly delimit the area of the subject matter explicitly addressing which assumptions are still waiting for a demonstration, beyond frail demonstrations of association or correlation.

Arterial stiffness is deemed to occur as a consequence of both biological ageing and arteriosclerosis[12]. Stiffened arteries require a greater amount of force to permit them to accommodate the stroke volume of the heart. The main consequence is an increase in pulse pressure which damages blood vessels in target organs such as the heart, brain or kidneys[13].

Measurement of aortic pulse wave velocity (PWV) is conceptually related to arterial stiffness[13]. By this acknowledgment, PWV, as a proxy of arterial stiffness, is credited to provide statistical evidence concerning the likelihood of occurrence of cardiovascular disease, and in some cases, of all cause mortality[13]. This notion has some epidemiological support: Regretfully, and surprisingly, no PWV measurement is currently recommended for any practical and accepted general use[14].

Imaging measures of fat liver content have considerable weaknesses[15] given that

they do not have complete correspondence to histology: Nonetheless, they have a key role in medical practice, which is extensive and well appreciated, providing a suitable way for monitoring liver fatty content over time and after therapeutic interventions[16].

CURRENT EVIDENCE

Current therapeutic evidence supports the recommendation of addressing changes toward healthier lifestyles, including diet and physical exercise, in both ATH and NAFLD, even when defined by non-invasive methodology[15]. Although such lifestyle approaches are acknowledged as effective interventions, they are questionably demonstrated by few clinical trials[17]. Disappointingly, such studies do not appear to be fully adequate due to limitations of size, duration and methods[18]. Accordingly, seemingly, none is currently considered suitable for promoting definitive and worldwide shared guidelines[15].

Research studies on the "association between aortic stiffness and liver steatosis in morbidly obese patients" have been published in several journals recently. One of them[19], deserves greater consideration because it is based on a realistic anatomic measure of fat content, and because, despite its goal and careful design and development, it recognizes that any independent relationship between aortic stiffness and fatty liver are missing in the results achieved. Both conditions depend, as expected, on better known, and lasting, risk factors: Namely, arterial hypertension, increased body weight and diabetes[19]. Apart from these points, this study is very valuable because its reference is not only that an appropriate liver histology sampling was available, but also because the measure of aortic stiffness was done by a reliable imaging-dependent tool, using an appropriate distance-based velocity measurement. These relevant features are very well displayed and justified within the article[19], and its lack is a major flaw of previous studies. Such combined approach is an advance, since previous studies have been performed with similar purposes but with more biased, "qualitative" and "subjective" methods[20]. The effort made in better understanding and facing the challenge of these two frequently overlapping diseases, even in the specific subset of extremely obese people, as in this investigation, promotes a negative answer to the claim of a causative relationship between ATH and NAFLD, if any, and, more, to the inclusion of NAFLD as a suitable concurrent risk factor for ATH[19].

Investigations and publications, using very indirect ATH and fatty liver imaging to assess patients' risk, may have also the aim of facilitating future drug trials[20]. However, there are many pros and cons, and a matter of concern is the disproportionately big and continuous flood of scientific contributions[20]: This, by itself, may impair an appreciation of the usefulness of these procedures to be used. The core of this valuable debate is mostly concealed by a kind of implicit but not evidence-based acceptance of the diagnostic merit of such very indirect measurement tools[15]. Nonetheless, several researchers unexpectedly are converging towards some agreement for accepting them as sustainable reference and indexes of outcome[15].

Even in a relatively limited field of knowledge and science, such as medical approaches to the development of a mostly affluent disease, a warning regarding the misuse of methods, alleged paradigms and unsupported axioms is needed[21]. Actually, scientific research, both basic and applied, is a dynamic process, constantly evolving and changing perspective, with critical thinking being at the core of the scientific method. In their daily work, scientists of every field are urged to keep pace with a broad spectrum of available data[22]. Interaction and overlap between these two fields of research, basic and applied, may allow us to solve particular problems or questions, and both may enhance novel knowledge and information[22]. The twin partners, ATH and NAFLD, are only apparently straightforward and relatively simple topics, and their interdependence calls for definitions without biases. Actually, encompassing many prevalent diseases and being related to several and recognized risk factors and health determinants, the study of the mutual relationship between NAFLD and ATH should involve big data analytics approaches[23,24]. Regretfully, available studies are not sufficiently comprehensive, not including even part of the data actually available in the specific database. There is a general context that may have effects on both fatty liver- and obesity- and on arteriosclerosis and related heart, brain and kidney diseases. This context encompasses the environment at large[25], behavioral factors[26], quantity and quality of dietary intake[27], with consequent obesity or nutritional-related disturbance[28], sedentary life, stress, detrimental levels of physical activity, sleep deprivation and night shifts for work or leisure[29], and certainly many others. Despite these limitations, overall, the effects of well-addressed intervention are reported as beneficial[30-32].

In brief, regarding the mutual relationship of ATH and NAFLD, no element reasonably suggests, apart from quite naive statistics, that one condition affects the other directly, despite the attractive hypothesis that fatty liver in itself may cause vascular damage. In these matters, it is always unsafe to treat contemporaneous events as causation[33,34].

IMPLICATIONS, CHALLENGES AND BIASES

Within other backgrounds and perspectives, the scientific community is the silent bystander of determined, even unreasonable, anti-science strain and campaigns[21]. Rejection of scientific method as an objective tool that can generate universal knowledge is of great concern for scientists and health professionals[21]. This eschewal may originate from the idea that scientific reductionism is inherently limited in reaching understanding of complex problems and, namely, health and disease topics. The very relevant ongoing debate on the "misuse of statistical significance"[35], leading sometimes to discarding of genuine discoveries, may add further fuel to mistrust in science and medicine. Reciprocally, suggesting conjectural "mechanisms of disease", such as claiming the long shadow of NAFLD as a "risk factor", if not as a causative factor of arterial stiffness and ATH, is probably very much overreaching reality and is harmful for the scientific credence of this area of medicine. "As long as different positions are discussed scientifically, controversy has a chance to move forward"[22]. This is a very important commitment for the physician, the researcher, the research projects evaluator and the reviewer. No golden rule is available, but the use of "naive" methods, not sufficiently adequate for the purposes of the scientific questions, when detected as inappropriate or frankly biased, should be reasonably discouraged and discarded.

CONCLUSION

Disease study processes are often too rigid, we must agree. In fact, the aim is to collect sufficient evidence, best if coordinated in a convincing architecture, relationships and support, in ways very similar to the theory of change. In fact, the study of diseases often, as in our case, includes the quantitative observation of numerous data and of the changes that are determined with interventions, spontaneous or within clinical trials. In this sense it is acceptable that there are assumptions that are not strongly supported, but it is quite possible that these assumptions are inconsistent and misleading so that, when detected, must be included as biases or mistakes in the proposed rationale of the dependent intervention for change. Despite the verification that NAFLD and cardiovascular disease are strongly interrelated[36], current evidence is that NAFLD may be only a possibly useful indicator for flagging early arteriosclerosis[37], and not a likely causative factor. Greater sustainable contributions by precision medicine tools, *i.e.*, by validated bioinformatics approaches and expertise, are needed to allow consideration of any role of big data and how they should be managed[38]. Very valuable investigations are already available, providing new insights into the relationship of NAFLD, fibrosis and ATH[39]. Also the shared molecular basis for ATH and vascular disease and its molecular relationships with several related diseases, namely NAFLD and Alzheimer's disease, are the focus of frontiers research in very well planned and managed investigations[39,40]. Currently, pathway-based analysis are elucidating key molecular mechanisms underlying complex diseases addressing the joint effect and integrality as function unit of multiple genes by extensive studies exploring large-scale "-omics" data[41]. In the mind and perception of most people, what matters is if such information and measures may guide the daily choices of physicians and if they may empower the adherence of patients to prescriptions, therapeutic pathways or lifestyle choices. The answer may be yes, but this is more a real world practice affair than the conclusion of any existing or ongoing trial or of any computational statistics analysis and prediction. The methodology described above, as in most more traditional research investigating associations and relationships, alludes implicitly to the assertion that where shadows overlap, they appear darker: Which should mean better visible, if not "clearer". However, this is not necessarily true. Actually, something is problematic in this assertion, and, more important, we may argue that it is where shadows overlap, that may matter. The need to integrate more omics data with different ones, such as epigenetic or epidemiological data, by bioinformatics, is evident when dealing with mechanisms and processes involving long cascades of multiple biological pathways[42] (Figure 1).

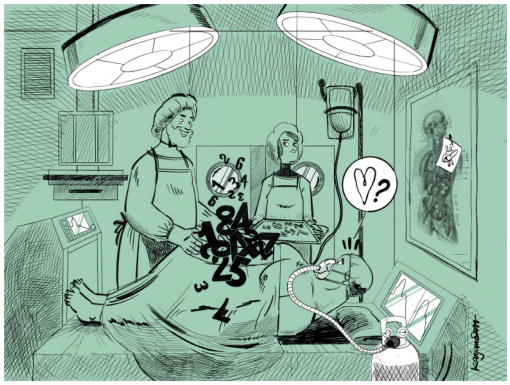

Figure 1 The naive and surreal "vision" of the artist displays in this drawing a tale of bodies and shadows. The researchers are exploring directly the liver of an obese patient, while the shadows of atherosclerosis and of arterial stiffness, *i.e.*, numbers, amazingly leak out from the liver and graphs of non-invasive measurements appear at the operating table's headboard. The theory that fatty liver in itself may hurt the vessels, as in the drawing of the anatomical drawing behind, lacks currently consistent supporting statistics. In these topics it is always unsafe to treat simultaneity as causation. The use of straightforward diagnostic methods for non-alcoholic fatty liver disease (surgical liver biopsy) and indirect measurements of aortic stiffness, pulse wave velocity, fails to demonstrate a direct independent relationship between the two conditions. The conjectural hypothesis of labeling non-alcoholic fatty liver disease as a novel risk factor for atherosclerosis, defined by the proxy of arterial stiffness, is far away to be demonstrated (drawing by Giuliano Cangiano-Kanjano).

The unpredictability of synergy among different pathways and the possibility of over-fitting, *i.e.*, the production of an analysis that corresponds too closely or exactly to a particular set of data, and may therefore fail to fit additional data or predict future observations reliably, must be appropriately considered when trying to illustrate the genetic, epigenetic, and environmental determinants of trigger and development of any disease[43]. Conceivably, when dealing with cross-talk of liver with ATH and fat cells[44], we should take into account what is actually overlapping, when, where, how and why. Mirroring the shadows of concepts, as we do using indirect or surrogate measures of ATH and of fatty liver, respectively, might be an unreliable and even misleading approach. Indeed, we are still waiting for high quality prospective studies in diverse racial-ethnic groups to further elucidate whether or not NAFLD is in fact causally related to ATH, while actual information provides some suggestion of limited associations[45,46]. The risk and the bias of a this clinical research tale are that, at last, some conclusion, as equally some intervention, based on uncertain assumptions might ultimately succeed in darkening or obscuring, certainly not enlightening concepts and mechanisms. The result will be weakening the strength of consequent warranted intervention which are still based on awareness and participatory behavioral changes[47] and on intervention models based on a theory of change[48], which usually is not clearly developed and monitored, as needed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Evaluation of bacterial biomarkers to aid in challenging inflammatory bowel diseases diagnostics and subtype classification

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Abstract

BACKGROUND

The challenges for inflammatory bowel disease (IBD) diagnostics are to discriminate it from gut conditions with similar symptoms such as irritable bowel syndrome (IBS), to distinguish IBD subtypes, to predict disease progression, and to establish the risk to develop colorectal cancer (CRC). Alterations in gut microbiota have been proposed as a source of information to assist in IBD diagnostics. *Faecalibacterium prausnitzii* (*F. prausnitzii*), its phylogroups, and *Escherichia coli* (*E. coli*) have been reported as potential biomarkers, but their performance in challenging IBD diagnostic situations remains elusive. We hypothesize that bacterial biomarkers based in these species may help to discriminate these conditions of complex diagnostics.

AIM

To evaluate the usefulness of indices calculated from the quantification of these species as biomarkers to aid in IBD diagnostics.

METHODS

A retrospective study of 131 subjects (31 controls (H); 45 Crohn's disease (CD), 25 ulcerative colitis (UC), 10 IBS, and 20 CRC patients) was performed to assess the usefulness of bacterial biomarkers in biopsies. Further, the performance of biomarkers in faeces was studied in 29 stool samples (19 CD, 10 UC). Relative

Informed consent statement: Informed consent from the subjects was obtained before enrolment.

Conflict-of-interest statement: Aldeguer X is a consultant from AbbVie and has received honoraria for lectures, including services on speakers' bureaus, from AbbVie, MSD, Shire and Takeda. Aldeguer X, Serra-Pagès, M and Garcia-Gil J own shares in GoodGut S.L. López-Siles M, Garcia-Gil J, Aldeguer X and Martinez-Medina M own patent WO2017025617A1 concerning a Method for the detection, follow up and/or classification of intestinal diseases. The other authors have nothing to disclose.

Data sharing statement: Datasets available from the corresponding author at marga.martinez@udg.edu. Consent was not obtained from participants for data sharing, but the presented data are anonymized, and the risk of identification is low. No additional data are available.

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abundances of total *F. prausnitzii* (FP), its phylogroups (PHGI and PHGII), and *E. coli* (E) quantification were determined by qPCR. Loads were combined to calculate the FP-E index, the PHGI–E index and the PHGII-E index. Biomarkers accuracy to discriminate among conditions was measured by the area under the receiver operating characteristic curve (AUC).

RESULTS
In biopsies, FP-E index was good for discriminating IBS from CD (AUC = 0.752) while PHGII-E index was suitable for discriminating IBS from UC (AUC = 0.632). The FP-E index would be the choice to discriminate IBD from CRC, especially from all UC subtypes (AUC ≥ 0.875), regardless of the activity status of the patient. Discrimination between UC patients that had the longest disease duration and those with CRC featured slightly lower AUC values. Concerning differentiation in IBD with shared location, PHGI-E index can establish progression from proctitis and left-sided colitis to ulcerative pancolitis (AUC ≥ 0.800). PHG I-E index analysis in tissue would be the choice to discriminate within IBD subtypes of shared location (AUC ≥ 0.712), while in non-invasive faecal samples FP or PHGI could be good indicators (AUC ≥ 0.833).

CONCLUSION
F. prausnitzii phylogroups combined with *E. coli* offer potential to discriminate between IBD and CRC patients and can assist in IBD subtypes classification, which may help in solving IBD diagnostics challenges.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Diagnostic tests; *Faecalibacterium prausnitzii*; *Escherichia coli*; Irritable bowel syndrome; Colorectal cancer

Core tip: This manuscript evaluates the usefulness of new indexes calculated from the quantification of *Faecalibacterium prausnitzii*, its phylogroups, and *Escherichia coli* as biomarkers to assist in challenges of inflammatory bowel disease diagnostics. Firstly, discrimination between inflammatory bowel disease and other intestinal disorders was tested. We present indices to distinguish colorectal cancer from inflammatory bowel disease, especially from subjects with ulcerative colitis. This is of significance given the association between chronic inflammation and the risk of colorectal cancer. In contrast, the proposed indices featured limited performance for discriminating inflammatory bowel disease from irritable bowel syndrome. Secondly, we approach if these biomarkers would be useful to discriminate within inflammatory bowel disease subtypes. We show here good biomarkers to differentiate inflammatory bowel disease subtypes of shared disease location, which may assist in monitoring the risk of progression of the inflamed area. Their application in non-invasive faecal samples is also demonstrated.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory bowel disorders of unknown aetiology that follow a course with periods of activity or flare-ups and periods of remission[1-4]. Crohn's disease (CD) and ulcerative colitis (UC) are the main idiopathic IBD[5-7]. Despite these disorders differing in location, histology, and distribution of inflamed areas, sometimes they feature overlapping clinical and pathological characteristics that hamper a distinct classification[8,9]. It is essential to discriminate both entities to establish an appropriate treatment strategy[10]. Besides,

there are other intestinal disorders, such as irritable bowel syndrome (IBS), that share symptoms similar to those observed in the early stages of IBD thus increasing its likelihood of misdiagnosis[11,12]. In contrast, chronic inflammation can lead to tumour formation and promote colorectal cancer (CRC) development. It would, therefore, be interesting to have a biomarker for IBD-progression to CRC, but currently, there is a lack of tools to predict which cases may progress to CRC. Altogether, current IBD diagnostics challenges are to discriminate phenotype variations within IBD accurately, but also to differentiate IBD from other gut conditions with milder or worsening phenotypes.

Given the absence of pathognomonic features, IBD diagnosis currently involves a comprehensive examination of the patient that includes clinical, endoscopic, radiologic, and histological criteria. Besides, as clinical manifestations of IBD are unstable during the disease course, a long monitoring period is needed to classify the disease phenotype accurately[11,15]. As IBD patients feature an imbalanced gut microbial community in comparison to healthy subjects[16-25], in the last years the implementation of bacteria representative of this dysbiosis as biomarkers has been started to be explored as a novel strategy to support IBD diagnostics and/or prognostics[23,26-30].

We and others have pointed out that the abundance of faecal or mucosa-associated *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a potential biomarker to discriminate between gut disorders[23,26-30] Moreover, *F. prausnitzii* in conjunction with *Escherichia coli* (*E. coli*) abundance (FP-E index) has been proven to be a better biomarker than total *F. prausnitzii* alone[26,29]. Besides, the quantification of *F. prausnitzii* phylogroups I (PHG I) and II (PHGII) has been proposed as a source of additional information to discriminate between IBD subtypes. However, the usefulness of an index using the quantification of the phylogroups in conjunction with *E. coli* remains to be explored. Also, there is a lack of comparative studies from a methodological aspect that would allow the establishment of the biomarker of choice.

It is against this background that we examined six options for biomarkers (*F. prausnitzii*, the two phylogroups or the combination of these three with *E. coli*) in a cohort of non-IBD controls (H), IBS, IBD and CRC subjects, to (1) establish which would be the best parameter to discriminate IBD patients from H and IBS subjects; (2) determine which would be the best parameter to discriminate IBD from CRC patients; and (3) identify which would be the most accurate parameter to discriminate within IBD subtypes by location. We hypothesize that bacterial biomarkers based in these species may be of help to discriminate these conditions of complex diagnostics.

MATERIALS AND METHODS

Patients, clinical data and sampling

In this study, data from two groups of subjects were included. Firstly, biomarker performance was tested in biopsy samples. We hypothesized that given the inflammatory nature of IBD, to look for biomarkers in the tissue would be strongly associated with disease course. Secondly, the usefulness of selected biomarkers was assessed in non-invasive samples (*i.e.*, stools).

To test the performance of the mucosa-associated bacterial biomarkers, a re-analysis of the data from a Spanish cohort including IBD, IBS, CRC, and H was performed (Table 1). Subjects were consecutively recruited by the Department of Gastroenterology at the Hospital Universitari Dr. Josep Trueta (Girona, Spain) and the Gastroenterology Unit at the Hospital Santa Caterina (Institut d'Assistència Sanitària of Girona, Salt, Spain) between May 2009 and November 2010. Patients were gender- and age-matched, except CD patients who were significantly younger than those in the H and IBS groups ($P < 0.001$) (Table 1). During routine endoscopy, up to three biopsy samples per patient were taken from different locations along the gut (Table 2) following standard procedures.

To test the performance of bacterial biomarkers in faecal samples, a cohort consisting of 29 IBD (19 CD and 10 UC) patients was recruited by the Gastroenterology Services of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) between March 2014 and May 2015. Subjects were age- and gender-matched for both the groups (Table 1). Participants were asked to collect a stool sample from one bowel movement in a sterile faecal collection container. Subjects brought samples to the hospital, where they were stored at −80°C until DNA extraction was performed.

To control bias between centres, patients with IBD were diagnosed according to standard clinical, pathological, and endoscopic criteria and categorized according to the Montreal classification. Patients with IBS were diagnosed according to Rome III criteria (available at http://www.romecriteria.org/criteria/). CRC diagnosis was

Table 1 Sample size and clinical characteristics of subjects

		Healthy[1]	Irritable bowel syndrome	IBD		Colorectal cancer	*P* value[3]
				Ulcerative colitis	Crohn's disease		
Cohort of subjects for biopsies samples collection	*n* (patients)	31	10	25	45	20	
	Age (mean ± SD, yr)	48.1 ± 16.3	42.4 ± 11.4	40.1 ± 15.8	33.5 ± 11.1	58.6 ± 7.52	< 0.001[4]
	Male, *n* (%)	16 (51.6)	2 (20.0)	16 (64.0)	26 (57.7)	14 (70.0%)	0.605[6]
	Active, *n* (%)	NA	NA	20 (80.0)	28 (62.2)	NA	0.100[5]
	Treatment, *n* (%)[2]						
	No treatment	NA	NA	16 (64.0)	17 (37.8)		NA
	Moderate immunosuppressant	NA	NA	3 (12.0)	17 (37.8)		NA
	Anti-TNFα	NA	NA	4 (16.0)	10 (22.2)		NA
	UC location, *n* (%)[2]						NA
	Ulcerative proctitis (E1)	NA	NA	6 (24.0)	NA	NA	
	Distal UC (E2)	NA	NA	11 (44.0)	NA	NA	
	Extensive UC or ulcerative pancolitis (E3)	NA	NA	6 (24.0)	NA	NA	
	CD location, *n* (%)[2]						NA
	Ileal-CD (L1)	NA	NA	NA	19 (42.2)	NA	
	Colonic-CD (L2)	NA	NA	NA	11 (24.4)	NA	
	Ileocolonic-CD (L3)	NA	NA	NA	14 (31.1)	NA	
Cohort of subjects for faecal samples collection	*n* (patients)			10	19		
	Age (mean ± SD, yr)			47.4 ± 18.3	43.5 ± 18.3		0.429[4]
	Male, n (%)			5 (50.0)	10 (52.6)		0.893[6]
	Active, *n* (%)[2]			1 (10)	7 (36.8)		0.185[5]
	Treatment, *n* (%)[2]	NA	NA	5 (50)	5 (26.3)	NA	
	No treatment	NA	NA	1 (10)	2 (10.5)	NA	
	Moderate immunosuppressant	NA	NA	3 (30)	12 (63.2)	NA	
	Anti-TNFα						
	UC location, *n* (%)[2]						NA
	Distal UC (E2)			3 (30.0)	NA		
	Extensive UC or ulcerative pancolitis (E3)			7 (70.0)	NA		
	CD location, *n* (%)						
	Ileal-CD (L1)			NA	10 (52.6)		NA
	Colonic-CD (L2)			NA	3 (15.8)		
	Ileocolonic-CD (L3)			NA	6 (31.6)		

[1]Controls consisted of subjects who underwent colonoscopy for different reasons: 9/31 rectal bleeding, 11/31 colorectal cancer familial history and 11/31 abdominal pain.
[2]Maximal disease extent at the time of sampling was available in 23/25 UC patients (cohort for biopsy samples), 4/10 UC patients (cohort for faecal samples), 44/45 CD patients. Activity status in the cohort that provided faecal samples was available for 4/10 UC patients and 11/19 CD patients. Treatment at sampling for the fecal sample's cohort was recorded for 9/10 UC patients, and for biopsy sample's cohort in 23/23 UC and 44/45 CD participants.
[3]Groups were compared by non-parametric statistical tests, and *P* value ≤ 0.05 was considered significant.
[4]Kruskal-Wallis.
[5] Mann-Whitney *U* test or
[6]χ^2 test as required. IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; CRC: Colorectal cancer; TNF: Tumour necrosis factor; NA: Not

applicable.

established by colonoscopy and biopsy examination, and none of the subjects underwent radiotherapy, chemotherapy or surgery. The control group consisted of subjects with normal colonoscopy who underwent this procedure for different reasons (Table 1). Clinically relevant data of all participants, such as age, gender, and disease activity at sampling, were collected (Table 1). Active CD were defined as those with CDAI > 150 whereas active UC patients had a Mayo score > 3.

Individuals included in this study were > 18 years old, did not have any other intestinal disease, and were not pregnant. Antibiotic treatment within the last month prior to sample collection was the only exclusion criterion. None of the subjects received probiotics before sample collection.

Sample treatment, DNA extraction, and qPCR assays

For biopsies, sample treatment and DNA extraction were performed as reported previously[26,27]. For faeces, 200-500 mg of faecal material were used for bacterial DNA extraction and purification with the NucleoSpin® Soil (Macherey-Nagel) and following the instructions from the manufacturer.

Previously designed and optimized 16S rRNA gene-targeted primers and probes were used for total *F. prausnitzii*[26], phylogroups, *E. coli* and total bacterial quantification using quantitative polymerase chain reaction (qPCR). Human cell numbers were determined with the control kit RT-CKFT-18S (Eurogentec, Belgium) according to the manufacturer's instructions.

Amplification reactions were performed as described elsewhere[26,27,35,36]. In brief, quantifications were performed in a total volume of 20 µL reactions containing: 1× TaqMan Universal PCR Master Mix 2× (Applied Biosystems, Foster City, CA, United States), 900 nmol/L of each primer, 300 nmol/L of each probe, and up to 50 ng of genomic DNA template. Samples were run in duplicate in the same plate. For data analysis, the mean of the duplicate quantifications was used. Duplicates were considered valid if the standard deviation between quantification cycles (C_q) was <0.34 (*i.e.*, a difference of < 10% of the quantity was tolerated). Quantification controls consisting of at least 5 reactions with a known number of target genes were performed to assess inter-run reproducibility. For samples with undetected values during quantification, the number of 16S rRNA gene copies equivalent to the detection limit of each reaction was used. Inhibition of total *F. prausnitzii* quantification was controlled by adding 10^3 copies of an internal amplification control (IAC) template to each reaction. It was considered that there was no inhibition if the obtained C_q was < 0.34 from those obtained when quantifying the IAC alone for any of the replicates. A non-template control (consisting of a reaction without *F. prausnitzii* DNA) and a non-amplification control (which did not contain any DNA template, either bacterial or IAC) were also included in each run. Negative controls resulted in undetectable C_q values in all cases.

All quantitative PCRs were performed using a 7500 Real Time PCR system (Applied Biosystems). The thermal profile was: a first step at 50 ºC during 2 min for amperase treatment, followed by a 95 ºC hold for 10 min to denature DNA and activate Ampli-Taq Gold polymerase, and a further 40 cycles consisting of a denaturation step at 95 ºC for 15 seconds followed by an annealing and extension step at 60 ºC (or at 64ºC for phylogroups quantification) for 1 min. Data were collected and analysed using the 7500 SDS system software version 1.4 (Applied Biosystems). All quantifications were performed under average PCR efficiencies of 89.51 ± 7.06%.

Sample size, data normalization and statistical analysis

The sample size was defined after the number of patients analysed in similar studies of bacterial abundance in subjects suffering of these conditions[20,22,26,28].

Relative abundances of total *F. prausnitzii*, phylogroups, and *E. coli* copy numbers were calculated by normalizing each species load for the total bacterial 16S rRNA gene copies. Data are given as the log10 of the ratio between 16S rRNA gene copies of the target microorganism and millions of total bacterial 16S rRNA genes detected in the same sample.

For biopsies, species relative abundances were combined to calculate the FP-E index as previously reported[26]. Similarly, the PHGI–E index and the PHGII-E index were calculated as follows:

PHGI-E index = $[\log_{10}(PHGI/Hc) - \log_{10}(E/Hc)] / [\log_{10}(TB/Hc)]$

PHGII-E index = $[\log_{10}(PHGII/Hc) - \log_{10}(E/Hc)] / [\log_{10}(TB/Hc)]$

Being PHGI and PHGII the 16S rRNA gene copies of *F. prausnitzii* phylogroup I or II respectively; E the 16S rRNA gene copies of *E. coli*; Hc a million of human cells; and

Table 2 Biopsy samples by conditions and locations

	No. Patients	No. biopsies				
		Terminal ileum	Transverse colon	Rectum	Unknown region	Total
H	31	14	24	10	0	48
IBS	10	1	3	3	12	19
CRC	20		3	17	0	20
UC	25	11	23	16	0	50
Location						
Ulcerative proctitis (E1)	6	4	5	5	0	14
Distal UC (E2)	11	3	11	8	0	22
Extensive UC or ulcerative pancolitis (E3)	6	3	6	1	0	10
CD	45	16	31	16	0	63
Location						
Ileal-CD (L1)	19	5	13	7	0	25
Colonic-CD (L2)	11	6	7	4	0	17
Ileocolonic-CD (L3)	14	4	10	4	0	18

H: Healthy controls; IBS: Irritable bowel syndrome; CRC: Colorectal cancer; UC: Ulcerative colitis; CD: Crohn's disease.

TB a million of 16S rRNA gene copies of total bacteria.

For faecal samples, as no Hc quantification was performed to normalize sample size, indexes were calculated as:

FP-E index = $\log_{10}$ (total *F. prausnitzii*/E)

PHGI-E index = $\log_{10}$ (PHGI/E)

PHGII-E index = $\log_{10}$ (PHGII/E)

Differences in categorical variables such as gender were assessed by the χ^2 test. For continuous variables such as age or biomarkers load, data normality was assessed through the KolmogorovâSmirnov test. The non-parametric Kruskal-Wallis test was used to asses differences in variables with more than two categories, such as diagnostics, and CD or UC disease location. Pairwise comparisons of subcategories of these variables were analysed using a Mann-Whitney *U* test. This test was also used to compare, within a subgroup of patients, variables with two categories.

The receiver operating characteristic curve analysis, a plot of the true-positive rate (sensitivity) versus false-positive rate (1-specificity), was applied to establish the usefulness of *F. prausnitzii*, along with each phylogroup, alone or in conjunction to *E. coli* counts (FP-E index, PHGI-E index, and PHGII-E index) to distinguish different intestinal conditions. The accuracy of discrimination was measured by the area under the receiver operating characteristic curve (AUC). An AUC approaching 1 indicates that the test is highly sensitive and highly specific, whereas an AUC approaching 0.5 indicates that the test is neither sensitive nor specific. For the best cut-off value, specificity and sensitivity were established.

All the statistical analyses were performed using the SPSS 15.0 statistical package (LEAD Technologies, Inc.). Significance levels were established for P values ≤ 0.05.

The statistical methods of this study were reviewed by MSc. Oliver Valero Coppin from the Statistical Service at the Universitat Autònoma de Barcelona.

RESULTS

Discrimination of IBD from H and IBS

When considering all biopsy samples (Figure 1A), PHGI quantification was the most discriminative biomarker between H and IBD patients (AUC > 0.75). This can be attributed to higher load of PHGI in H in comparison to the other groups of subjects (Supplementary Table 1). Notably, discrimination was especially good between H and subjects with CD, which achieved 73% specificity and 91% sensitivity at the best cut-off value (Log_{10}[16S rRNA phylogroup I/10^6 16S rRNA total bacteria] =2.3). This discrimination was particularly accurate when analysing ileal samples (AUC > 0.9) (Figure 1B). Besides, discrimination between H and IBD subjects achieved greater AUC values when considering only active IBD patients (Figure 1C). However, the discrimination was still good (AUC > 0.75) when taking into account only those with inactive disease. Therefore, our results support PHGI as an indicator of healthy gut

status.

Regarding discrimination between IBD and IBS patients, different biomarkers performed best to distinguish IBS from UC or CD. When pooling all biopsy samples (Figure 1A), PHGII-E index was suitable to discriminate IBS from UC. This discrimination was excellent when considering ileal or rectal biopsies and suitable for colonic biopsies analyses (Figure 1B). It is of note that the PHGII-E index allowed good discrimination between these two conditions, even in the inactive cohort of patients (Figure 1C). In contrast, FP-E index was good for discriminating IBS from CD when pooling all samples, although this was not sustained for all sampled locations, probably due to the effect of the location of inflammation in CD. In contrast, FP was the best biomarker to discriminate IBS and CD in colonic and rectal samples, whereas PHGI counts discriminated best at the ileum (Figure 1B). This biomarker was good to discriminate IBS from active CD patients, whereas the PHGII-E index provided the best discrimination between IBS and inactive CD. Overall, to select a general biomarker to discriminate IBS from IBD, useful in all kinds of samples and conditions was challenging. However, FP could be an interesting candidate as performed in the suitable-excellent AUC range for all comparisons, regardless of the intestinal region selected for analysis.

Discrimination between IBD with colonic inflammation and CRC

In general, the FP-E index was the most discriminatory between CRC and IBD patients when taking into account all biopsy samples together (Figure 2A), because lower FP-E values were associated with CRC subjects (Supplementary Table 1). Notably, discrimination was especially good between CRC and UC patients, which achieved 85% specificity and 94% of sensitivity at the best cut-off value (FP-E index= 0.009). This discrimination was excellent between CRC and patients with ulcerative proctitis (E1) and ulcerative pancolitis (E3) with 85% specificity and 100% sensitivity, while for patients with extensive UC (E2) sensitivity was reduced to 86% with the same specificity rate. Although good discrimination was achieved (AUC > 0.870) we observed that discern between E2 patients and those with CRC featured slightly lower AUC values, and in turn, these groups of patients had the longest disease duration (mean years of disease duration ± SD by UC subtype was: E1 = 0.93 ± 1.69; E2 = 7.10 ± 4.27; E3 = 2.63 ± 2.20.

In addition, this excellent discrimination was sustained regardless of the activity status of the patients. Regarding the location of sample (Figure 2B), for our particular cohort, colonic biopsies were the most discriminatory between CRC and those patients with E1 and E3, although good separation of groups was also achieved with rectal samples. In turn, rectal samples performed better to discriminate between E2 subjects and those with CRC.

The FP-E index was also suitable to classify CRC patients and those with CD of colonic location (*i.e.*, C-CD and IC-CD). Interestingly, better AUC values were obtained for PHGI and when considering rectal samples alone, which needs further confirmation given the low number of samples analysed at this location for CD patients.

Discrimination within IBD with shared location

Biomarkers analyses in biopsy samples: The FP-E index was the best biomarker to differentiate UC from CD patients considering all locations (Figure 3A), given that UC patients had higher FP-E index values than CD patients (Supplementary Table 1). However, no consensus could be reached about the biomarker that performed best when comparing IBD subtypes with shared location of the inflammation. PHGI-E index was good to differentiate E1 and E2 from E3, particularly in ileal samples (AUC ≥ 0.875) although suitable discrimination was also obtained when analysing colonic biopsies. In contrast, the PHGII-E index was the most accurate to discriminate C-CD from all UC locations when considering all samples together, and this was sustained in colonic samples (Figure 3B).

As regards discrimination between CD locations, in general, all the biomarkers showed AUC ≤ 0.75 except for the PHGII-E index in ileal samples, which allowed for good discrimination between IC-CD from both C-CD and I-CD.

Interestingly, when considering only active patients, the PHGI-E index was the most discriminatory for all the comparisons when pooling samples, and when considering only those from the ileum and colon. Except for CD with ileal involvement, also suitable discrimination was obtained with the PHGI-E index from rectal samples. Analyses in inactive patients are not shown because, in most cases, they could not be conducted given the low number of samples with these characteristics when separating by IBD subtype.

Biomarkers analyses in faecal samples: Analyses of IBD faecal samples showed that

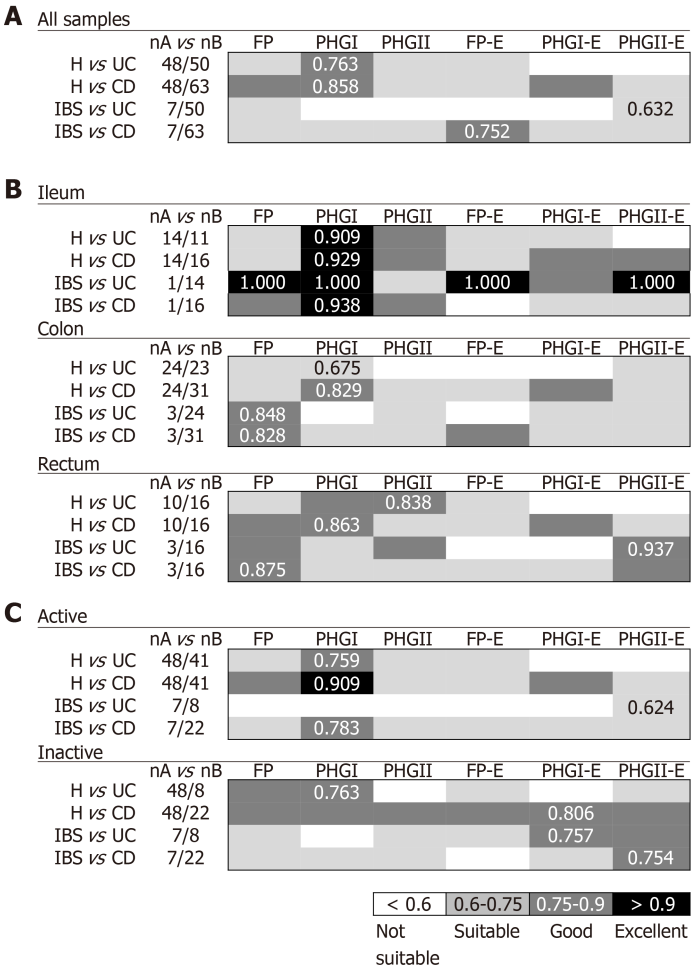

Figure 1 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate between milder gut conditions [Healthy controls (H) and irritable bowel syndrome] and inflammatory bowel disease (ulcerative colitis and Crohn's disease) by pooling all biopsy samples together (A), by location of sampling (B) and by activity status (C). Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CD: Crohn's disease; IBS: Irritable bowel syndrome.

the most suitable biomarker to discriminate between UC and CD conditions was PHGI (Figure 3C), whose load was higher in the former, regardless of the disease extent (Supplementary Table 2). This biomarker was different from that found in biopsies, and the AUC was 1.4 times lower than that obtained in tissue samples.

In contrast, better AUC values were achieved in faecal samples for PHGI and PHGI-E compared to those in biopsies to discriminate C-CD from E2, E3 and IC-CD, although corroboration by engaging more C-CD subjects is needed. It is of note that the results obtained for FP as a biomarker to distinguish IC-CD from I-CD, which substantially improved the biopsy results.

DISCUSSION

Quantification of bacterial biomarkers may be a valuable tool to assist in the diagnosis of intestinal disorders. In this work, we explored the usefulness of two species (*E. coli* and *F. prausnitzii*), extensively reported as dysbiosis representatives of IBD[16,18-20,22,23,26-30], to discriminate between different gastrointestinal disorders.

Firstly, we explored whether or not these bacterial biomarkers could assist in discriminating IBD from IBS, where symptoms can be similar at early stages of the disease. It was observed that a general biomarker to discriminate IBS from IBD could not be established, and therefore two biomarkers should be used. While FP-E index allowed discrimination between IBS and CD, PHGII-E index was the most appropriate to discriminate between IBS and UC. Our cohort of IBS patients was limited and not classified by IBS subtypes. As differences in gut microbiota

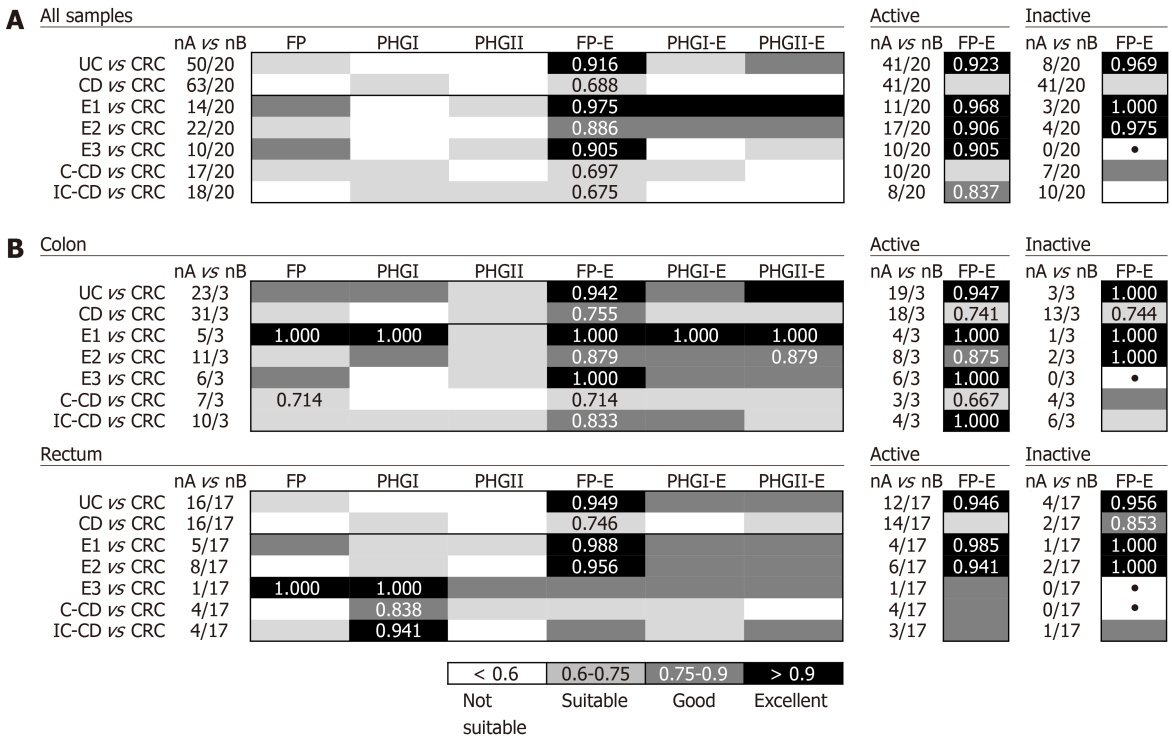

Figure 2 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate inflammatory bowel disease with colon inflammation and colorectal cancer by pooling all biopsy samples together (A) and by location of sampling (B). For the best biomarker, results depicted by activity status of the patients are shown in the right panels. Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. : AUC not calculated (comparisons with one empty group of subjects). E1: Ulcerative proctitis; E2: Distal UC; E3: Extensive UC or ulcerative pancolitis; C-CD: Colonic-CD; IC-CD: Ileocolonic-CD; FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CRC: Colorectal cancer; CD: Crohn's disease.

composition have been found between patients with diarrhoea-predominant IBS and those with constipation-predominant IBS[37], we propose that in further studies aiming to define a biomarker between IBS and IBD, phenotype should be taken into account. Besides, the inclusion of newly diagnosed patients would be of interest to establish whether these biomarkers would be of assistance to discriminate between conditions at an early stage of the disease, particularly when symptoms are overlapping.

Secondly, as there is an association between IBD (especially those involving colonic inflammation) and risk of CRC[13,38], the usefulness of the six biomarkers to tell apart CRC and IBD patients with colonic inflammation was explored. Among the six options of biomarkers considered, the FP-E index was the most discriminatory between CRC and IBD patients, especially from UC, regardless of the activity status of the patient and irrespective of whether colonic or rectal samples were used. This observation is of particular relevance because it has been demonstrated that the extent and duration of the disease increase the risk of patients with UC developing CRC. Future follow-up studies to establish if this index would be useful to predict the risk of CRC development associated with IBD are needed. In contrast, discrimination for CD patients was somewhat limited. Therefore it would be of interest to determine if the combination of *F. prausnitzii* or its phylogroups with other representatives of CRC dysbiosis enriched in CRC patients[39,40] such as some phylotypes related to *Bacteroides*, *P. stomatios* or *G. morbillorum* could provide a clearer diagnostic test.

Finally, the usefulness of these biomarkers to discriminate IBD subtypes with shared location of inflammation was assessed. The PHGI-E index was a good parameter to discriminate UC subtypes, which is of interest for clinicians to monitor the risk of progression of the inflamed area. From our data, this index allowed the best discrimination within UC subtypes with active disease in ileal and colonic samples. However, a deeper analysis to decipher which sample is the best to analyse is required, as in our study, not all the subjects provided samples from all locations, and interindividual variability may be affecting our observations. Also, further confirmation is required concerning inactive patients since our cohort was limited.

In contrast, we have observed that PHGII load, in conjunction with *E. coli* counts, can distinguish with suitable accuracy between all UC patients regardless of their disease subtypes and patients with colonic CD (C-CD). The capacity to discriminate between patients with C-CD and E3 is noteworthy because inflammation in these two

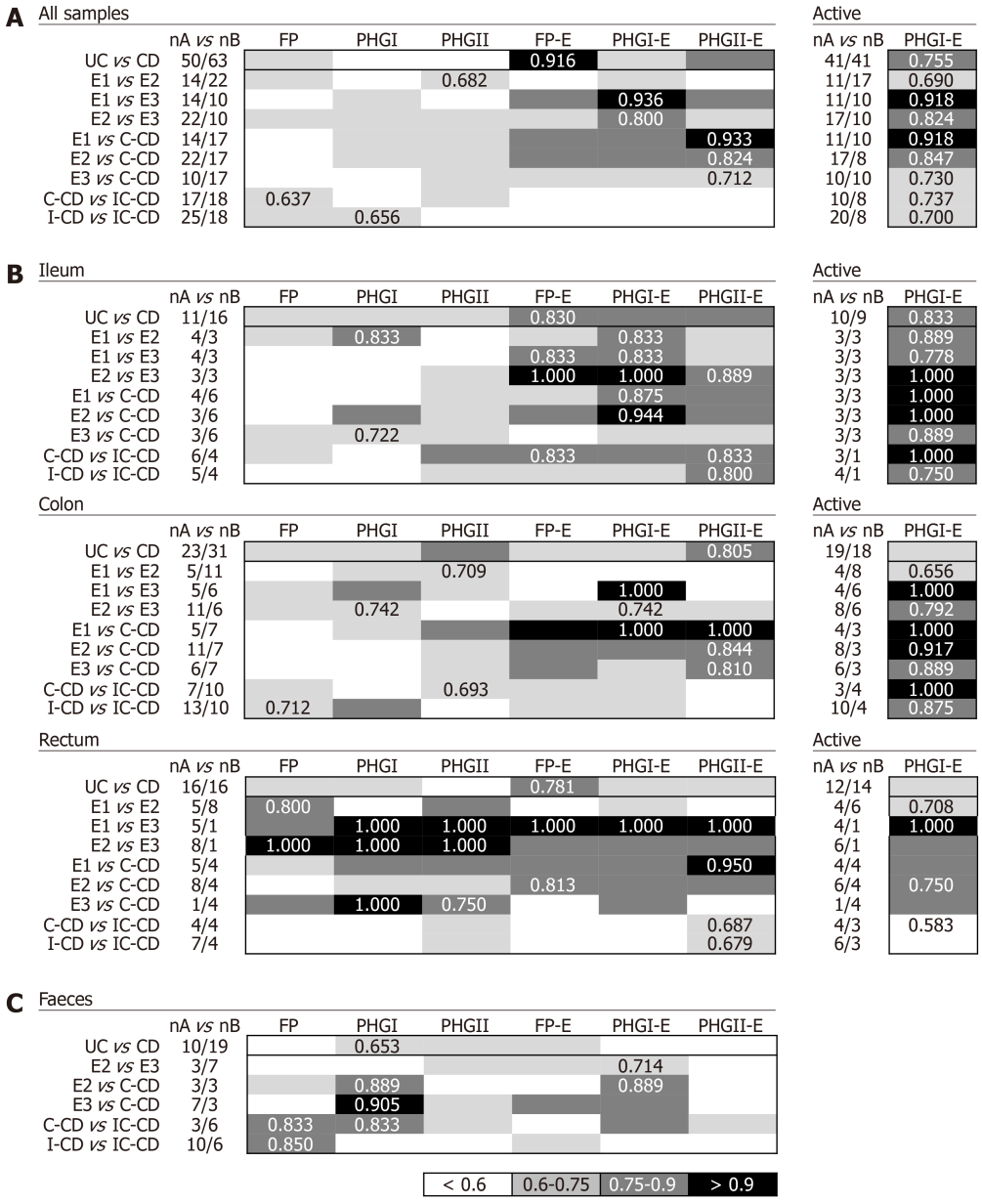

Figure 3 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate within inflammatory bowel disease with colon inflammation taking into account all biopsy samples together (A), by location of sampling (B) and faeces (C). For tissue samples, selected results for PHGI- *Escherichia coli* of active patients are shown in the right panels. Data for inactive patients is not included because of the small cohort engaged. Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. UC: Ulcerative colitis; CD: Crohn's disease; E1: Ulcerative proctitis; E2: Distal UC; E3: Extensive UC or ulcerative pancolitis; C-CD: Colonic-CD; IC-CD: Ileocolonic-CD; FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CRC: Colorectal cancer; CD: Crohn's disease.

disorders affects a wide area of the colon and may present similar clinical manifestations, thus hampering a clear classification. Due to differences in treatment and management between UC and CD[10] it is extremely important to discriminate between these two entities accurately.

The best discrimination for CD *vs* UC was obtained for patients without shared inflamed area (data not shown), but the discrimination needs to be improved to differentiate IBDs with shared disease location, particularly within CD. The combination of *F. prausnitzii* or its phylogroups with other representatives of IBD dysbiosis may be a way to improve discrimination between IBD subtypes. For instance, depletion of *Roseburia hominis* has been reported as representative of UC dysbiosis, while the depletion of *Ruminococcus gnavus* and *Ruminococcus torques*, with a concomitant increase in *Dialister invisus* or *Bifidobacterium adolescentis* have been reported as signatures of CD dysbiosis[16,18]. In addition, the identification of novel species whose abundance differs between IBD subtypes sharing the inflamed location may be of assistance in this regard.

Overall, we observed that the FP-E index would be the selected biomarker to

discriminate IBD from CRC while PHG I-E index would be the choice to discriminate within IBD subtypes, and yet no general biomarker of preference could be established to discriminate IBS from IBD. PHGII-E index would be suitable to tell apart IBS and UC patients, whereas the FP-E index could be of assistance to discriminate between IBS and CD although further confirmation on its usefulness for inactive patients is required. It has been reported that active CD and UC can be specifically diagnosed monitoring the faecal bacterial community in conjunction with leukocyte counts. Although in this previous study location of disease has not been considered, it demonstrates that serologic biomarkers may be a source of additional information. A recent study suggested that anti-*E. coli*, anti-*Fusobacterium nucleatum*, and anti-*F. prausnitzii* antibodies did not possess diagnostic value for CD or UC,. However, it would be worth testing if discrimination between gut conditions is enhanced when these bacterial indicators are combined with other previously reported serologic biomarkers of intestinal disease [such as calprotectin, lactoferrin, C-reactive protein, Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (p-ANCA), and Anti-*Saccharomyces cerevisiae* antibodies (ASCA)].

In order to establish if the proposed indices are suitable for discriminating conditions in less invasive samples, data from the quantification of these species in faeces of IBD subjects was used. We restricted the proof of concept to IBD subjects as these are the conditions more similar in clinical traits and therefore more difficult to discriminate. Our results allowed to demonstrate that, despite our initial selection of biomarkers was based in tissue samples, they are also valuable in faeces. However, differences in which biomarkers performed the best were found. These differences could be because the cohorts used for faecal and biopsy analyses involved different subjects or may reflect the fact that gut microbiota composition is different between faeces and biopsies. Thus, in the future, if biomarkers are selected from tissue, it is crucial to test performance in faecal samples, ideally including the two kinds of samples from the same subject. We have observed that whereas quantification of *E. coli* in biopsies improved discrimination, the role in improving discrimination when faecal samples are used remained more limited.

On the one hand, this may be explained by the fact that this species may be directly involved in host-interaction during diseases. On the other hand, this information leads us to hypothesize that for future applications, other biomarkers selected from faecal samples analysis could also be included. Concerning *F. prausnitzii*, the observed differences on which subpopulation should be used as a biomarker, may be related to the distribution of phylogroups along with the gastrointestinal tract, each one with specific metabolic features[43,44].

To robustly validate our observations would require a larger cohort of completely independent patients, including volunteers from different ethnicities, to test these biomarkers as a tool for gut disease diagnostics. Moreover, it would be of interest to test whether *F. prausnitzii* or its phylogroups, in conjunction with *E. coli* as biomarkers could discriminate other intestinal disorders within IBD such as indeterminate colitis, unclassified IBD, pouchitis, microscopic colitis, and diverticulosis as these can also be possible confounding conditions.

ARTICLE HIGHLIGHTS

Research background

Currently, inflammatory bowel diseases (IBD) diagnostics features several challenges mainly related to its accurate differentiation from other disease with similar symptoms. In the last years, some studies have shown that the abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a potential biomarker to discriminate between gut disorders. This species load in conjunction with *Escherichia coli* (*E. coli*) abundance (F-E index) has been proven to be a better biomarker than total *F. prausnitzii* alone. Besides, the quantification of *F. prausnitzii* phylogroup I and phylogroup II has been proposed as a source of additional information to discriminate within IBD. However, the usefulness of an index including the quantification of the phylogroups in conjunction with *E. coli* remains to be explored, and also its applicability to tell apart these conditions from other gut disorders with milder or worsen phenotypes.

Research motivation

Currently, IBD diagnosis involves a comprehensive examination of the patient that includes clinical, endoscopic, radiologic, and histological criteria. In addition, as clinical manifestations of IBD are unstable during the disease course, a long monitoring period is needed to classify the disease phenotype accurately. As IBD patients feature an imbalanced gut microbial community in comparison to healthy subjects, in the last years the implementation of bacteria representative of this dysbiosis as biomarkers has been started to be explored as a novel strategy to support IBD diagnostics and/or prognostics.

Research objectives

The main objective of this study was to evaluate six options of bacterial biomarkers in terms of their capability to discriminate IBD from other gut disorders and within IBD subtypes.

Research methods

Adult males and females undergoing routine colonoscopy at the Hospital Dr. Josep Trueta and Parc Hospitalari Martí i Julià in Girona (Spain) were asked to participate, providing either biopsy and/or faecal samples. Subjects included healthy controls as well as patients with IBD, CRC or irritable bowel syndrome (IBS). Genomic DNA extracts of samples were used to assess the load of bacterial markers candidates (total *F. prausnitzii*, phylogroup I and II of this species and *E. coli*) by qPCR using specific primers previously reported. Relative abundances to total Bacteria present in the sample, and indices combining *F. prausnitzii* and *E. coli* were calculated. Biomarkers accuracy to discriminate conditions was measured by the area under the receiver operating characteristic curve (AUC). To the best of our knowledge, this is the first study that tests combination of *F. prausnitzii* phylogroups and *E. coli* application to assist in discriminating challenging IBD diagnostic conditions, compares their performance with previously reported biomarkers and further corroborates results in non-invasive samples.

Research results

This study reveals that the F-E index would be the choice to discriminate IBD from colorectal cancer (CRC), especially from ulcerative colitis (UC), regardless of the activity status of the patient and irrespectively if a colonic or a rectal sample was used. This observation is of particular relevance because there is an association between IBD (especially those involving colonic inflammation) and the risk of CRC. Besides, we have observed that PHG I-E index is a good parameter to differentiate pancolitis from other UC subtypes, which is of interest for clinicians to monitor risk of progression of the inflamed area. The application of bacterial biomarkers in feces is also demonstrated, which is a non-invasive method and may represent a step forward to implement these biomarkers in clinical practice to support IBD diagnostics.

Research conclusions

This study corroborates that *F. prausnitzii* combined with *E. coli* can help to discriminate within IBD subtypes both in tissue and fecal samples, as well as offer potential to differentiate IBD and CRC patients. Use of biopsy samples presented better performance, but we confirmed that suitable results in fecal samples were shown too. The comparison of the performance of new indices with those previously reported in the literature has allowed establishing the biomarker of choice to select depending on the conditions to discriminate. From these comparisons, we hypothesize that given the complexity of the disease in terms of multiple subtypes and phenotypes during the disease course, it would be complicated the establishment of a universal biomarker using only two species and total microbiota composition could be a more informative approximation in this regard. However, given the outcome obtained only with the biomarkers evaluated here, we envisage that implementation of bacterial load assessment in clinical routine may ease IBD diagnostics in the future, for example for initial screening.

Research perspectives

This study contributes to providing evidence that bacterial biomarkers assessment may help in solving intestinal disorders diagnostic challenges. Because differences in performance were observed between tissue and faecal samples, attention should be paid to this issue in similar studies. Future directions of research could assess if discrimination between gut conditions is enhanced when these bacterial indicators are combined with other bacterial or serologic biomarkers of intestinal disease. Also, validation in a larger cohort of completely independent patients, including volunteers from different regions would be required to define a tool with worldwide application in clinical routine.

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MINIREVIEWS

Correlations of morphology and molecular alterations in traditional serrated adenoma

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Abstract

Traditional serrated adenoma was first reported by Longacre and Fenoglio-Presier in 1990. Their initial study described main features of this lesion, but the consensus diagnostic criteria were not widely adopted until recently. Traditional serrated adenoma presents with grossly protuberant configuration and pinecone-like appearance upon endoscopy. Histologically, it is characterized by ectopic crypt formation, slit-like serration, eosinophilic cytoplasm and pencillate nuclei. Although much is now known about the morphology and molecular changes, the mechanisms underlying the morphological alterations are still not fully understood. Furthermore, the origin of traditional serrated adenoma is not completely known. We review recent studies of the traditional serrated adenoma and provide an overview on current understanding of this rare entity.

Key words: Traditional serrated adenoma; Serrated polyps; *KRAS*; *BRAF*; Colon

Core tip: This mini-review summarizes recent findings of traditional serrated adenoma. The origin of traditional serrated adenoma and its molecular pathogenesis are discussed in details.

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INTRODUCTION

Colorectal carcinoma (CRC) is a heterogeneous disease in terms of its molecular pathways of carcinogenesis. Most, if not all CRCs, arise from conventional adenomas or serrated lesions, the latter accounting for 5%-35% of CRC[1,2]. Serrated polyps include hyperplastic polyps (HPs), sessile serrated lesions (SSLs), traditional serrated adenomas (TSAs) and unclassified serrated adenomas according to 2019 World Health Organization classification of colonic epithelial neoplasms[3]. Sessile serrated lesions and TSAs are regarded as precursors to CRC, while small HPs are considered to have little risk for neoplastic progression. TSAs comprise 0.56%-1.9% of colorectal polyps and are the least characterized serrated lesions in the colorectal carcinogenesis[4-6]. Endoscopically, TSAs show exophytic protuberant configuration and pinecone-like appearance[7,8]. Histologically, TSAs feature architectures of complex filiform or villiform growth pattern, slit-like or flat-top serration and ectopic crypt formation (ECF) which is defined as small rudimental crypts located on the side of villous structure and displaced from bottom muscularis mucosa. Cytologically, TSA is characterized by lining of epithelium with abundant eosinophilic cytoplasm, pseudostratified pencillate nuclei and dispersed chromatin (Figure 1)[9]. Although it is debatable which feature is the most sensitive and which one is the most specific, it is agreed that none of them alone is sufficient or required for diagnosing TSA. Fulfilling 2 out of 3 core features (ECF, eosinophilic cytoplasm and slit-like serration) may be more reproducible in making the diagnosis of TSA[10].

The molecular pathogenesis of TSA is poorly understood due to its rarity. Less is known about the mechanism that drives precursor lesions and their subsequent risk of progression. In this review, we will present the currently available literature, focusing on the origin of TSA. We will also attempt to correlate the molecular changes with morphologic features, which might help us understand how TSAs develop from precursor lesions or *de novo*.

ORIGIN OF TSA

TSAs are probably underdiagnosed by pathologists for several reasons. TSAs are the rarest among the three serrated colonic polyps, comprising of about 5% serrated polyps and 0.56%-1.9% of all colorectal polyps[4-6], and widely accepted consensus criteria for diagnosing TSA were not available until recently. Chetty[11] listed a constellation of architectural and cytological features of TSA in a succinct review of the entity. However, none of these features are unique or specific for TSA. The minimal criteria for diagnosing TSA are also not specified in many studies. Additionally, TSAs are often admixed with HP or SSL[8,12,13], causing difficulty in recognition. Three variants of TSA were described, the prototypical filiform TSA, the less common flat TSA[12] and the rare mucin-rich TSA[9].

Genetic heterogeneity of TSAs contributes to the variation in cytomorphology. Almost 90% of TSAs develop through two mutually exclusive pathways: *BRAF* mutation (56.4%) and *KRAS* mutation (31.9%)[8,12-15] (Table 1). The remaining 10% may have other pathways involved such as EGFR (Figure 2)[16] that appears to segregate with *KRAS*-mutated polyps[12]. *BRAF* gene encodes an anti-apoptotic serine-threonine kinase. *BRAF* V600E activating mutation is an early event that drives serrated lesion into CRC[17,18]. TSAs with *BRAF* mutation often show a flat growth pattern with serrated dysplasia, high CpG island methylator phenotype (CIMP) and are more likely located in the proximal colon than *KRAS*-mutated TSA[8,12]. *KRAS*-mutated TSA are usually distally located and exophytic with adenomatous dysplasia. In addition to *KRAS* mutation, TSAs from distal colon show selective methylation of *SMOC1* gene and loss of its expression, which are also frequently associated with high-grade adenoma and CIMP-low/microsatellite stable CRC[19].

TSAs may arise from precursor lesions of microvesicular HP or SSL or may occur *de novo*. *BRAF* mutated TSAs are also more likely admixed or associated with HP or SSL-like lesions, which are identified in TSA in 38%-52.3% of cases[8,12,20]. One early study suggested that serrated precursor lesions adjacent to distal TSA are distinguished from SSL by lack of Annexin A10 despite shared morphologic and molecular features[21]. Annexin A10 is normally expressed in upper gastrointestinal tract[22]. It is identified as a marker of SSL[23] and is expressed in colorectal cancer of serrated pathway undergoing gastric programming[24]. Thus, it is not surprising that the serrated precursor lesions of TSA in this study, arising predominantly from distal colon, are distinctive from proximal colonic SSL[21]. It is more likely that small flat TSAs identified in proximal colon would be expressing Annexin A10. More recently, Bettington and colleagues compared small polyps (< 1 cm) (71% from the distal colon)

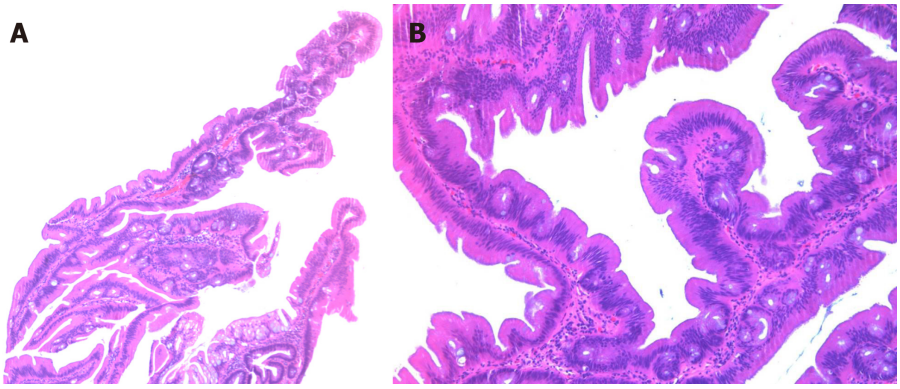

Figure 1 Low and high power view of the traditional serrated adenoma. A: A low power view (40×) of the traditional serrated adenoma shows viliform growth of the polyp with slit-like serration; B: A high power view (100×) demonstrates ectopic crypt formation, eosinophilic cytoplasm and pencillate nuclei.

and shoulder lesion in large TSAs, demonstrating similar immunophenotypic and molecular profiles[20]. These findings support that small TSAs do exist and may arise at least partially from some HP/SSL-like precursors.

WNT signaling is the main driver of colon cancer and physiological proliferation of colonic crypts[25]. Alterations in components of WNT pathway including mutations of *RNF43*, *APC* and *CTNNB1*, and overexpression of *RSPO* (due to fusion gene or amplification), can all lead to stabilization and nuclear localization of β-catenin and activation of WNT signaling[26]. Nuclear β-catenin staining, as well as p53 positivity, loss of p16 and *MLH1* promoter methylation is seen in the late development of polyps with dysplastic features[12,13]. However some molecular studies showed that components of WNT signaling are frequently altered in TSAs (30%-70%) regardless of the degree of dysplasia[15,27]. A recent study using microdissection to interrogate genetic changes revealed a stepwise molecular change in TSAs and associated precursors[28]. Clonally, the HP/SSL-like precursors share the identical mitogen-activated protein kinase (MAPK) pathway gene mutations (*BRAF* or *KRAS*) with TSAs. However, these precursors exhibit fewer mutated *WNT* pathway genes or heterozygotic mutations (*i.e.*, *RNF43*, *APC*, and *CTNNB1*) than TSA with biallelic inactivation. This study supports the sequence of MAPK to WNT alterations in TSA developing from HP and SSL-like lesions (Figure 2). One drawback of this study is that only one out of 15 polyps had *KRAS* mutation. Hence TSAs with *KRAS* mutation, which are predominantly found in the distal colon and typically are large in size, remain of uncertain in terms of origin and critical molecular alterations during development.

HISTOLOGIC-MOLECULAR CORRELATIONS

The presence of histologic features in TSAs is highly variable depending on their size and location. ECF is considered relatively more specific whereas slit-like serration and typical cytology are more sensitive features (Table 1). Serration is the common feature of HP, SSL and TSA. However, the cytomorphology of these three entities differs, reflecting distinct mechanisms underlying their development. *BRAF* or *KRAS* are two initiating mutations commonly seen in serrated polyps, activating MAPK pathway. HPs are characterized by saw-toothed serration in the upper half to third of the crypts and absence of basal crypt dilation[29,30]. Epithelial proliferation with defective apoptosis[5], delayed crypt cellular migration and maturation toward the surface leads to infolding of epithelial lining and formation of HPs. The majority of HPs are innocuous, largely because *KRAS* mutation in HP does not expand the stem cell pool but instead increases transit-amplifying cells in the mid and upper regions of crypts[31].

By contrast, SSLs have irregular proliferative zones and bidirectional maturation toward both surface and base of the crypt, causing pathognomonic basal crypt dilation and lateral spread of crypt base[32]. This architectural change was suggested to be similar to gastric foveolar growth pattern characterized by a mid-level proliferative compartment and bidirectional differentiation[11]. Another salient feature of SSLs is prominent inhibition of apoptosis in contrast to HPs and TSAs[33].

Compared to HP and SSL, TSAs have slit-like, flat-top serration rather than saw-toothed serration. Eosinophilic cells in TSA with luminal brush border and a

Table 1 Histologic and molecular changes in traditional serrated adenoma

Country/Territory	Polyp	Distal	*BRAF*	*KRAS*	Wild type	ECF	Slit-like serration	Typical cytology	Ref.
United States	24	96% (23)	29% (7)	46% (11)	25% (6)	NA	NA	79% (19)	[14]
South Korea	107	74.8% (80)	55.1% (59)	33.6% (36)	11.2% (12)	79.4% (85)	100% (107)	100% (107)	[8]
Taiwan	60	61.7% (37)	35% (21)	52% (31)	13.3% (8)	NA	NA	NA	[13]
Australia	200	71% (142)	67% (134)	22% (43)	11% (23)	89% (178)	98% (196)	100% (200)	[12]
Japan	129	82.2% (106)	61.2% (79)	34.8% (45)	3.9% (5)	NA	NA	NA	[15]
Australia	70	71% (50)	47% (33)	31% (22)	21% (15)	67% (47)	81% (57)	NA	[20]
Total	590	74.2% (438)	56.4% (333)	31.9%(188)	11.7% (69)	82% (310/377)	95% (360/377)	98.5% (326/331)	

Numbers in parenthesis are case numbers. ECF: Ectopic crypt formation; NA: Not applicable.

prominent villiform growth pattern are the features reminiscent of small intestine morphology[9]. It was believed that eosinophilic cytoplasm seen in TSA is due to cellular senescence[32]. Senescence and apoptosis are two protecting approaches of cells and tissue in response to oncogenic stresses[34]. They are the barriers that must be overcome in precursor lesions to promote and progress into fully developed TSAs. In TSAs, depending on locations, *BRAF* or *KRAS* are the initiating mutations activating MAPK pathway. Both, however, may cause cellular senescence and cell cycle arrest through p53/p21 axis or p16INK4 activation[29,34]. SSL is also well known to have high rate of *BRAF* mutation[18]. Therefore, it is not uncommon to observe occasional eosinophilic atypical cells in SSLs[32]. Animal models supported that *BRAF* V600E mutation causes cellular senescence after first wave of proliferation[35] and a shift of balance from proliferation to differentiation, which can be rescued by a loss of additional differentiation-promoting factors (CDX2, SMAD4 and p16) or activation of WNT signaling[36]. In SSLs and TSAs located in proximal colon, hypermethylation of *P16INK4* promoter and loss of p16 expression are the late events[12] that may cause evasion of senescence program implemented by *BRAF* mutation, whereas activation of WNT is likely the pathway employed for the progression of distally located TSAs.

ECF is a key feature of TSAs, especially in large protuberant ones in the distal colon. The presence of ECF in TSAs ranges from 67% to 89%, depending on the location and size of the polyps[8,12,20]. ECF is defined as small crypts displaced from muscularis mucosa, likely representing a progression step by disrupting the signalings of colonic crypt homeostasis. Bone morphogenetic protein (BMP4) signaling is probably a good candidate. BMP signaling plays an important role in villus morphogenesis and is known to promote cell differentiation and repress crypt formation[37,38]. Studies using human pluripotent stem cells demonstrated that BMP signaling is only transiently required for colonic differentiation, while small intestinal differentiation is the default program in the absence of BMP signaling[10]. Loss of BMP signaling in animal model[37] leads to ectopic crypt foci that resembles the phenotype of juvenile polyposis syndrome, which is known to harbor *BMPR1A* and *SMAD4* mutations in human[39,40]. Therefore, it is possible that ECF and villiform growth of TSA represent dysplastic transformation of colonic crypts into small intestinal villous morphology. This morphology may arise owing to aberrant molecular pathways such as BMP signaling, which controls villus-crypt homeostasis of gastrointestinal tract. Along with early events of *BRAF* or *KRAS* mutation, additional molecular changes drive proliferation of intestinal epithelium and shape them into TSA with distinct cytomorphology. Further accumulations of aberration in p53 and WNT signaling lead to progression of TSA into prominent dysplasia and CRC.

CONCLUSION

TSAs are rare serrated polyps located predominantly in the distal colon. At least two pathways have been identified, converging on activation of MAPK by *BRAF* or *KRAS* mutations. Small HP/SSL-like lesions with *BRAF* mutations might initiate as TSA precursors. Whether it is the same case occurring in the small serrated lesion with *KRAS* mutation awaits further investigation. Because TSA-derived colorectal cancer is considered very aggressive[30], study of the TSA-carcinoma sequence, its progression from lack of dysplasia to high-grade atypia and malignancy, is also warranted in the future.

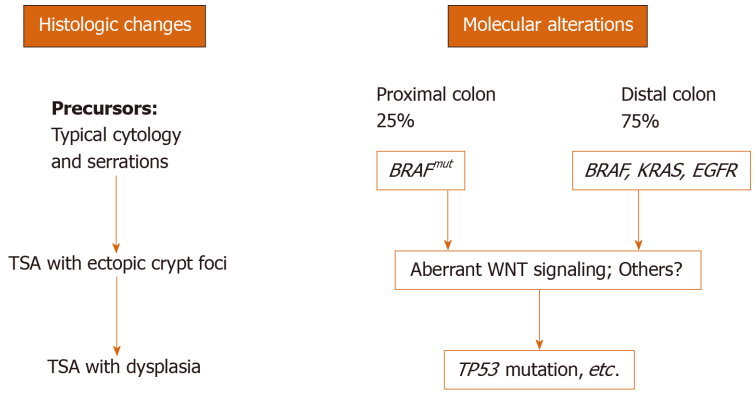

Figure 2 Histologic changes of traditional serrated adenoma parallel molecular alterations. During traditional serrated adenoma (TSA) development, mutations in *BRAF* (*BRAF*^{mut}), *KRAS* and *EGFR* cause typical cytomorphology and serration in precursor lesions. Accumulation of molecular alterations such as aberrant WNT signaling leads to fully developed TSA. Other pathways (Bone morphogenetic protein?) in addition to WNT signaling might also be involved in this step. Finally, mutations such as *TP53* will cause the progression of TSA into high-grade dysplasia and malignant transformation.

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ORIGINAL ARTICLE

Basic Study

P2X7 receptor antagonist recovers ileum myenteric neurons after experimental ulcerative colitis

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Abstract

BACKGROUND

The P2X7 receptor is expressed by enteric neurons and enteric glial cells. Studies have demonstrated that administration of a P2X7 receptor antagonist, brilliant blue G (BBG), prevents neuronal loss.

AIM

To report the effects of BBG in ileum enteric neurons immunoreactive (ir) following experimental ulcerative colitis in *Rattus norvegicus albinus*.

METHODS

2,4,6-trinitrobenzene sulfonic acid (TNBS group, $n = 5$) was injected into the distal colon. BBG (50 mg/kg, BBG group, $n = 5$) or vehicle (sham group, $n = 5$) was given subcutaneously 1 h after TNBS. The animals were euthanized after 24 h, and the ileum was removed. Immunohistochemistry was performed on the myenteric plexus to evaluate immunoreactivity for P2X7 receptor, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), HuC/D and glial fibrillary acidic protein.

RESULTS

The numbers of nNOS-, ChAT-, HuC/D-ir neurons and glial fibrillary acidic protein-ir glial cells were decreased in the TNBS group and recovered in the BBG group. The neuronal profile area (μm^2) demonstrated that nNOS-ir neurons decreased in the TNBS group and recovered in the BBG group. There were no differences in the profile areas of ChAT- and HuC/D-ir neurons.

CONCLUSION

Our data conclude that ileum myenteric neurons and glial cells were affected by

Committee on Animal Use of the Biomedical Science Institute of the University of São Paulo. Furthermore, all protocols were approved by the Ethics Committee on Animal Use of the Biomedical Science Institute of the University of São Paulo (Protocol 68/2016).

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ulcerative colitis and that treatment with BBG had a neuroprotective effect. Thus, these results demonstrate that the P2X7 receptor may be an important target in therapeutic strategies.

Key words: P2X7 receptor; Brilliant blue G; Myenteric plexus; Experimental ulcerative colitis; Ileum; Chemical coding

Core tip: This work aims to analyze the effects of experimental ulcerative colitis (EUC) in ileum myenteric neurons immunoreactive (ir) for P2X7 receptor, neuronal nitric oxide synthase, choline acetyltransferase, HuC/D and enteric glial cells immunoreactive for glial fibrillary acidic protein. The animals were treated with P2X7 receptor antagonist, brilliant blue G (BBG). The results showed that the numbers of neuronal nitric oxide synthase-, choline acetyltransferase-, HuC/D-ir neurons and glial fibrillary acidic protein-ir glial cells were decreased in the EUC group and recovered in the animals treated with BBG. BBG treatment demonstrated that the P2X7 receptor may be a possible therapeutic target in the treatment of the EUC.

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INTRODUCTION

The enteric nervous system (SNE) performs functions in gastrointestinal tract motility, control of gastric acid secretion, regulation of fluid movement through the epithelium, changes in local blood flow, and interactions with the endocrine and intestinal immune systems[1,2]. This system has two ganglionic plexuses, the myenteric plexus and the submucosal plexus. The myenteric plexus is located between the outer longitudinal muscular layer and the circular muscle layer, extending throughout the digestive tract from the esophagus to the rectum. The submucosal plexus is found predominantly in the small and large intestines and has a smaller ganglion, and its interconnected fibers are thinner compared to those of the myenteric plexus[1,2]. Enteric glial cells have functions to support neurons, regulate synaptic transmission, and release cytokines[3,4].

Inflammatory bowel diseases (IBDs) are disorders that affect the digestive tract. These problems include ulcerative colitis and Crohn's disease[5]. There are changes in SNE populations in ulcerative colitis of animals and humans[6-10]. Additionally, McCready *et al*[11] described the expansion of the inflammatory process from the distal ileum.

Studies have shown that the release of ATP by enteric neurons as noncholinergic and nonadrenergic neurotransmitters may be related to intestinal motility[12-15]. Purinergic receptors are classified into P1 and P2, where P1 receptors are activated by the adenine nucleoside and P2 receptors are activated by the nucleotide ADP (adenosine diphosphate) or ATP[16]. P2X receptors are ion channels with selective permeability to Ca^{2+}, K^+ and Na^+ cations, and they can be found in the central nervous system, ENS and enteric glial cells[15,17,18]. Seven types of P2X receptors have been described: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7[18-21].

The P2X7 receptor has been described in the ENS[22,23]. Studies show that brilliant blue G (BBG) is a P2X7 antagonist, and its low toxicity[24,25] and high selectivity make this compound an ideal candidate to block the adverse effects of P2X7 receptor activation[26]. P2X7 receptor-deficient animals have been shown to exhibit improvements in their overall condition when subjected to experimental ulcerative colitis[27]. Peng *et al*[28] demonstrated recovery of the rat spinal cord after mechanical injury following BBG administration. Additionally, Palombit *et al*[29] observed recovery of BBG-treated enteric neurons following an ischemia and reperfusion protocol.

This work aims to analyze the effects of experimental ulcerative colitis in neurons immunoreactive (ir) for neuronal nitric oxide synthase (nNOS), choline

acetyltransferase (ChAT) which is marker for intrinsic primary afferent neurons (IPANs) and excitatory motor neuron, and HuC/D (a pan-neuronal marker) and enteric glial cells immunoreactive for glial fibrillary acidic protein (GFAP) in the ileum in animals treated with BBG.

MATERIALS AND METHODS

The animal experiments in this study were conducted according to the current regulations of the Ethics Committee on Animal Use of the Biomedical Science Institute of the University of São Paulo. Furthermore, all protocols were approved by the Ethics Committee on Animal Use of the Biomedical Science Institute of the University of São Paulo (Protocol 68/2016). Young male Wistar rats (200–300 g body weight) were maintained under standard conditions at 21 °C with a 12-h light-dark cycle. All groups were supplied with water *ad libitum*.

Ulcerative colitis induction

The rats were anesthetized with a mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) administered subcutaneously. Inflammation was induced through the intrarectal insertion of a polypropylene 8 cm cannula. 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma, Saint Louis, United States) was injected at a dose of 30 mg/kg in 600 μL of 30% ethanol in the colon lumen (n = 5). Sham animals (n = 5) were injected with vehicle. BBG (50 mg/kg, Sigma Aldrich, United Kingdom, n = 5) or saline was injected 1 h following TNBS injection (n = 5)[28,29]. The survival time after colitis induction was 24 h.

For macroscopic and microscopic analyses, colitis was assessed according to macroscopic colonic injury[30]. The scores were stratified as follows: 0 = normal, 1 = presence of hyperemia without ulcers, 2 = ulcerations without hyperemia, 3 = ulcerations at one site, 4 = two or more sites of ulcerations, 5 = sites of damage extending > 1 cm, and 6 to 10 = sites of damage extending > 2 cm, with the score increasing by 1 for each additional cm[30]. The microscopic colitis scores were assessed using a scoring system adapted from Erdogan *et al*[31] and Fabia *et al*[32]. The scores were categorized as follows according to the corresponding parameters. Ulcerations: 0 = no ulcer, 1 = single ulceration not exceeding the lamina muscularis mucosa, 2 = ulcerations not exceeding the mucosa, and 3 = ulcerations exceeding the submucosa. Edema (submucosa): 0 = no edema, 1 = mild edema, 2 = moderate edema, and 3 = severe edema. Inflammatory cell infiltration: 0 = no infiltration, 1 = mild infiltration, 2 = moderate infiltration, and 3 = dense infiltration.

The scoring of the disease activity index (DAI) was analyzed in the control, sham and colitis rats. Percentage weight change, stool consistency and/or presence of occult bleeding were examined. The scores were categorized as follows according to the corresponding parameters. Weight change (%) score: 0 = 1%, 1 = 1%-5%, 2 = 5%-10%, 3 = 10%-15%, and 4 = > 15%. Stool consistency (%) score: 0 = normal[a] (well-formed pellets), 1 = normal, 2 = loose stool[b] (pasty and semiformed stools that do not stick to the anus), 3 = loose stool, and 4 = diarrhea[c] (liquid stools that stick to the anus). Occult/gross rectal bleeding: 0 = normal, 1 = occult blood +, 2 = occult blood ++, 3 = occult blood +++, and 4 = gross bleeding. The disease activity index was calculated by summing the score parameters[7,33,34].

Immunohistochemistry

For immunohistochemistry, fresh segments of the ileum were dissected and placed in PBS containing nicardipine (10^{-6} mol/L, Sigma, United States) to inhibit tissue contraction. The segments were opened along the mesenteric border and cleaned with PBS. The tissues were then placed mucosal side down onto a sheet of balsa wood and fixed overnight at 4 °C with 4% paraformaldehyde in sodium phosphate buffer 0.2 mol/L (pH 7.3). The next day, the tissue was cleared of fixative with three 10-min washes in 100% dimethyl sulfoxide (DMSO), followed by three 10-min washes in PBS. All tissue was stored at 4 °C in PBS containing sodium azide (0.1%). The tissue collection was performed by the same researcher who placed the tissue onto the balsa board to be fixed (see material and methods). The processing maintained the same stretch between preparations.

The fixed tissue was subdissected to remove the mucosal and circular layers, producing only the longitudinal muscle layer with the myenteric plexus. For immunohistochemistry, the myenteric plexus of the ileum was preincubated with 10% normal horse serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature. The antibodies used in this study are listed in Table 1. Double labeling was achieved using combinations of the antisera indicated in Table 1. After incubation with primary antisera, tissues were washed three times for 10 min each time in PBS

and incubated with various secondary antibodies (Table 1). The PBS washes were repeated, and the tissue was mounted in buffered glycerol with 0.5 mol/L sodium carbonate (pH 8.6).

The stained tissue specimens were examined using a Nikon 80i fluorescent microscope. The images were captured using a digital camera and the NIS Nikon software package. Additionally, the tissue specimens were analyzed using confocal microscopy on a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. Images were taken at 512 × 512 pixels, and the thickness of each optical section was 0.5 μm. Z-stacks of ir cells were captured as a series of optical sections with a center spacing of 0.2 μm. The confocal images were collected using LSM 5 Image Zeiss processing software and were further processed using Corel Photo Paint and Corel Draw software.

Histological analysis

Samples of ileum and distal colon from the sham (n = 3), TNBS (n = 3) and BBG (n = 3) groups were washed in PBS, opened at the mesenteric border, placed on balsa wood and fixed in 4% paraformaldehyde for 48 h. The tissues were treated in increasing concentrations of alcohol, cleared in xylene and embedded in Paraplast Plus® (Sigma). The tissues were cut (5 mm) and stained with hematoxylin-eosin (HE). Qualitative analysis was performed to observe changes caused by experimental ulcerative colitis. For analyzes it was used a Nikon 80i microscope coupled to a camera with NIS-Elements AR 3.1 (Nikon) software.

Quantitative analysis

The analyzes were also done by double marking the membrane preparations on the Nikon 80i fluorescence microscope. First, the neurons were located by the presence of the fluorophore that marks an antigen and then the filter was changed to determine whether or not the neuron was marked by a second antigen, located by a second fluorophore of a different color. The cohort size was 100 neurons, and the data were collected from preparations obtained from five animals. The percentages of double-ir neurons were calculated and expressed as the mean ± SE (n = number of preparations). In total, 100 neurons and 100 enteric glial cells from each membrane preparation were analyzed from each of the sham (n = 5), TNBS (n = 5) and BBG (n = 5) groups[7,29]. The density of neurons (neurons/cm^2) ir for P2X7, nNOS, ChAT, anti-HuC/D (pan-neuronal marker) and GFAP (pan-glial cells) as well as the neuronal morphological profiles was measured by analyzing all of the samples at 100 × magnification. Counts were made in 40 microscopic fields (0.000379 cm^2) for each antigen in a zig-zag pattern to avoid counting the same area more than once for each antigen in each animal, and a total of 200 microscopic fields were analyzed per immunoreactivity. Cell profile areas (μm^2) were obtained for 100 randomly selected neurons in two whole-mount preparations per animal per nNOS, ChAT, anti-HuC/D immunoreactivity assay from 5 rats for each group. A total of 500 neurons per group were analyzed using a Nikon 80i microscope coupled to a camera with NIS-Elements AR 3.1 (Nikon) software and were measured using Image-Pro Plus software version 4.1.0.0. Data were compared by analysis of variance (ANOVA) and Tukey's test for multiple comparisons, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

On histological analysis, the ileum showed no lesions and had a normal appearance in the sham, TNBS and BBG groups (Table 2). However, the histological observations showed that in distal colon the edema and inflammatory cell infiltration in the TNBS group. The mucosa, the circular and longitudinal muscles and the distal colon enteric neurons in the Sham and BBG groups were preserved. Additionally, the microscopic scores did not indicate ulcerations, edema or inflammatory cell infiltration in the ileums of all groups (Table 2).

The DAI showed changes in weight (%), stool consistency (%) and occult/gross rectal bleeding (%) in the sham, TNBS and BBG groups (Figure 1). The results show that there was an increase in DAI scores, stool consistency and occult bleeding in the TNBS group and a recovery in the BBG group. Histological studies revealed that the mucosa, lamina propria and submucosal ganglia in all groups were not affected (Figure 2).

Immunohistochemical analysis showed that the P2X7 receptor was present in the myenteric neurons in the sham, TNBS and BBG groups. P2X7 receptor-ir neurons were labeled for HuC/D, nNOS, ChAT and GFAP in all groups studied (Figures 3, 4, 5 and 6). The P2X7 receptor immunoreactivity colocalized 100% with neurons positive for HuC/D, nNOS, ChAT and GFAP in all groups.

Table 1 Characteristics of primary and secondary antibodies

Antigen	Host	Dilution	Source
P2X7 receptor	Rabbit	1:200	Millipore (AB5246)
nNOS	Sheep	1:2000	Millipore (AB1529)
ChAT	Goat	1:50	Chemicon (AB144P)
Anti-HuC/D	Mouse	1:100	Molecular probes (A-21271)
GFAP	Rabbit	1:400	DAKO (Z0334)
GFAP	Mouse	1:200	Sigma (G3893)
Secondary antibodies			
Donkey anti-rabbit IgG 488		1:500	Molecular probes (A21206)
Donkey anti-sheep IgG 594		1:100	Molecular probes (A11016)
Donkey anti-mouse IgG 594		1:200	Molecular probes (A21203)

nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; GFAP: Glial fibrillary acidic protein.

GFAP-positive glial cells were observed close to ChAT- and nNOS-immunoreactive neurons (Figures 7 and 8).

P2X7 receptor immunoreactivity per area of neurons decreased in the TNBS group by 10.6% compared to that in the sham group ($P < 0.05$). There was an increase of 20.4% in the BBG group compared to the TNBS group ($P < 0.01$) (Figure 9A).

nNOS-positive neurons per area decreased by 22.9% in the TNBS group compared to the sham group ($P < 0.05$). An increase of 22.2% was observed in the BBG group compared to the TNBS group ($P < 0.01$) (Figure 9B).

The ChAT-immunoreactive neurons per cm^2 were reduced by 34.0% in the TNBS group compared to the sham group ($P < 0.05$), and they were increased by 13.9% in the BBG group compared to the TNBS group ($P < 0.01$) (Figure 9C).

HuC/D-immunoreactive neurons per area reduced by 15.4% in the TNBS group compared to the sham group ($P < 0.05$). Furthermore, there was an increase of 19.5% in the BBG group compared to the TNBS group ($P < 0.01$) (Figure 9D).

The GFAP-positive enteric glial cells per cm^2 reduced by 14.4% in the TNBS group compared to the sham group ($P < 0.05$), and there was an increase of 17.7% in enteric glia in the BBG group compared to the TNBS group ($P < 0.01$) (Figure 9E).

Regarding neuronal profile area, the nNOS profile area decreased by 12% in the TNBS group compared to the sham group ($P < 0.05$), and an increase of 8% was observed in the BBG group compared to the TNBS group ($P < 0.05$) (Figure 10A). No differences were observed between the ChAT- and HuC/D neuronal profile areas of the studied groups. Due to colocalization, the profile areas of P2X7-positive nerve cells in the myenteric plexus were not quantified (Figure 10).

The distribution of nNOS-positive neurons showed that the size ranged from 50 μm^2 to 1050 μm^2 and that 22% to 25% of neurons were between 150 μm^2 and 350 μm^2 in the sham, TNBS and TNBS groups (Figure 11A).

The immunoreactive ChAT neurons demonstrated that the range size ranged from 50 μm^2 to 950 μm^2, with 27% to 37% being between 150 μm^2 and 250 μm^2 in all groups (Figure 11B).

The distribution of HuC/D-positive neurons showed that the size ranged from 50 μm^2 to 850 μm^2, with 21% to 43% between 150 μm^2 and 250 μm^2 in all groups (Figure 11C).

DISCUSSION

The experimental ulcerative colitis model affected ileum myenteric plexus neurons, and these neurons recovered with the use of BBG. The colitis model established by injecting TNBS in ethanol solution is considered effective and is widely used in the literature to produce experimental ulcerative colitis[10,35]. From the DAI, it was possible to observe that the experimental ulcerative colitis affected the weight, change in stool consistency and occult/gross rectal bleeding, and the reduction in these parameters showed an improvement in the condition of the group treated with BBG.

In our work, macroscopic and microscopic analysis of the ileum did not show that the mucosa or submucosa were affected. However, the literature has shown that experimental ulcerative colitis presents superficial inflammation, limited to mucosal and submucosal regions in the distal colon[6,7,36].

Table 2 Macroscopic and microscopic scores of ileums from the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups

Variables	Sham	TNBS	BBG
Macroscopic score	0	0	0
Microscopic score			
Ulcerations	0	0	0
Edema (submucosa)	0	0	0
Inflammatory cell infiltration	0	0	0

For macroscopic and microscopic analyses colitis was assessed according to colonic injury[30]. The scores were stratified as follows: 0 = normal, 1 = presence of hyperemia without ulcers, 2 = ulcerations without hyperemia, 3 = ulcerations at one site, 4 = two or more sites of ulcerations, 5 = sites of damage extending > 1 cm, and 6 to 10 = sites of damage extending > 2 cm, with the score increasing by 1 for each additional cm[30]. The microscopic colitis scores were assessed using a scoring system adapted from Erdogan *et al*[31] and Fabia *et al*[32]. The scores were categorized as follows according to the corresponding parameters. Ulcerations: 0 = no ulcer, 1 = single ulceration not exceeding the lamina muscularis mucosa, 2 = ulcerations not exceeding the mucosa, and 3 = ulcerations exceeding the submucosa. Edema (submucosa): 0 = no edema, 1 = mild edema, 2 = moderate edema, and 3 = severe edema. Inflammatory cell infiltration: 0 = no infiltration, 1 = mild infiltration, 2 = moderate infiltration, and 3 = dense infiltration[31,32]. TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

Enteric neurons of the distal colon are affected by experimental ulcerative colitis and Crohn's disease[37-39]. In our study, we observed a decrease in nNOS, ChAT and HuC/D-positive neurons in the ileum, thus demonstrating that experimental colitis in the distal colon may affect neurons in locations distant from the origin of the lesion.

Immunohistochemical studies have demonstrated the expression of P2X7 receptors in the SNE[6,7,22]. Gulbransen *et al*[40] observed activation of P2X7 receptors during colitis. In our study, we observed P2X7 receptor immunoreactivity in ileum myenteric plexus cells in all groups. We also observed a reduction in the number of P2X7 receptor immunoreactive cells in the TNBS group compared to that in the sham group and recovery of the neurons in the BBG group. da Silva *et al*[6,7] observed a decrease in P2X7 receptor-positive cells in the distal colon following experimental ulcerative colitis.

Purinergic mechanisms may be involved in the etiology of many conditions that affect the nervous system, due the large extracellular release of ATP[41]. Changes in purinergic receptor expression in neurons are observed in neuronal maturation, differentiation, acute CNS injuries, such as hypoxia ischemia, mechanical stress and inflammation.

It was observed that experimental ulcerative colitis affected neuronal classes in the ileum. In this study, a decrease in nNOS-, ChAT- and HuC/D-immunoreactive neurons per μm^2 was observed, and the recovery of these neurons per μm^2 was observed with BBG treatment. It has been observed that several classes of enteric neurons are affected in Crohn's disease and experimental ulcerative colitis[8,38,39,42]. Studies have demonstrated that ischemia and reperfusion decrease the number of enteric neurons[23] and treatment with BBG, a P2X7 antagonist, recovers rat enteric neurons[29]. Additionally, transient receptor potential channel vanilloid 2 (TRPV2) and the release of nitric oxide (NO) are related to intestinal motility[43].

Enteric glial cells have different functions in the face of gastrointestinal disorders[44,45]. In our study, we observed a decrease in GFAP immunoreactive glia in the ileum of the TNBS group compared to that in the sham group and recovery in the BBG group.

The evaluation of morphometric changes (measurement of cell profile area) has been widely studied in several experimental protocols. In the present work, the analysis of the profile of the nNOS-, ChAT- and HuC/D-ir neurons was performed to determine changes in the profile areas in these neuronal classes. However, only nitrergic neurons of the TNBS group showed a decrease, and those treated with BBG demonstrated recovery. The increased neuronal profile area could be explained as a compensatory mechanism due to neuronal death in the TNBS group[6,29].

The literature elucidates the neuroprotective role of P2X7 receptor blockade, as well as a possible increase in the expression of anti-inflammatory factors IL-10 and TGF-β1 by KO P2X7 mice, expressly helping to control inflammation[27,29,46].

Studies have described that gastrointestinal epithelia release ATP and Transient Receptor Potential Vanilloid 4 (TRPV4) is expressed throughout the gastrointestinal epithelia[47].

The importance of this work is to demonstrate that experimental ulcerative colitis

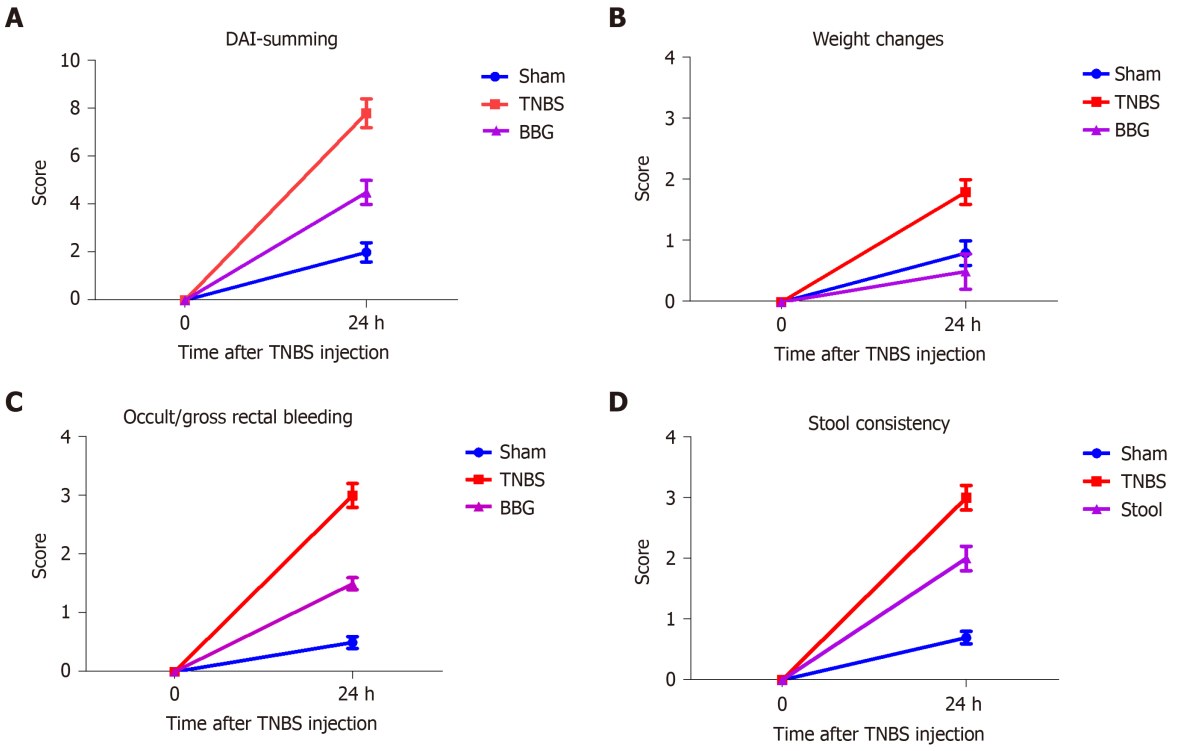

Figure 1 Scoring of the disease activity index in the in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A: The disease activity index was calculated by summing the score parameters: Mild activity was classified from 1 to 4; moderate activity, from 5 to 8; and maximal activity from 9 to 12. The scores were categorized as follows according to the corresponding parameters; B: Weight change score: 0 ≤ 1, 1 = 1-5, 2 = 5-10, 3 = 10-15, and 4 ≥ 15%; C: Occult/gross rectal bleeding: 0 = normal, 1 = occult blood +, 2 = occult blood ++, 3 = occult blood +++, and 4 = gross bleeding; D: Stool consistency score: 0 = normal (well-formed pellets), 1 = normal, 2 = loose stool (pasty and semiformed stools that do not stick to the anus), 3 = loose stool, and 4 = diarrhea (liquid stools that stick to the anus). DAI: Disease activity index; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

affects distant organs such as ileum myenteric neurons that express the P2X7 receptor. In addition, BBG treatment was shown to be effective in the recovery of ileum myenteric neurons, thus demonstrating that the P2X7 receptor may be a possible therapeutic target in the treatment of the effects of experimental ulcerative colitis. Also, has been described that the expansion of the inflammatory process from the distal neck to the distal ileum.

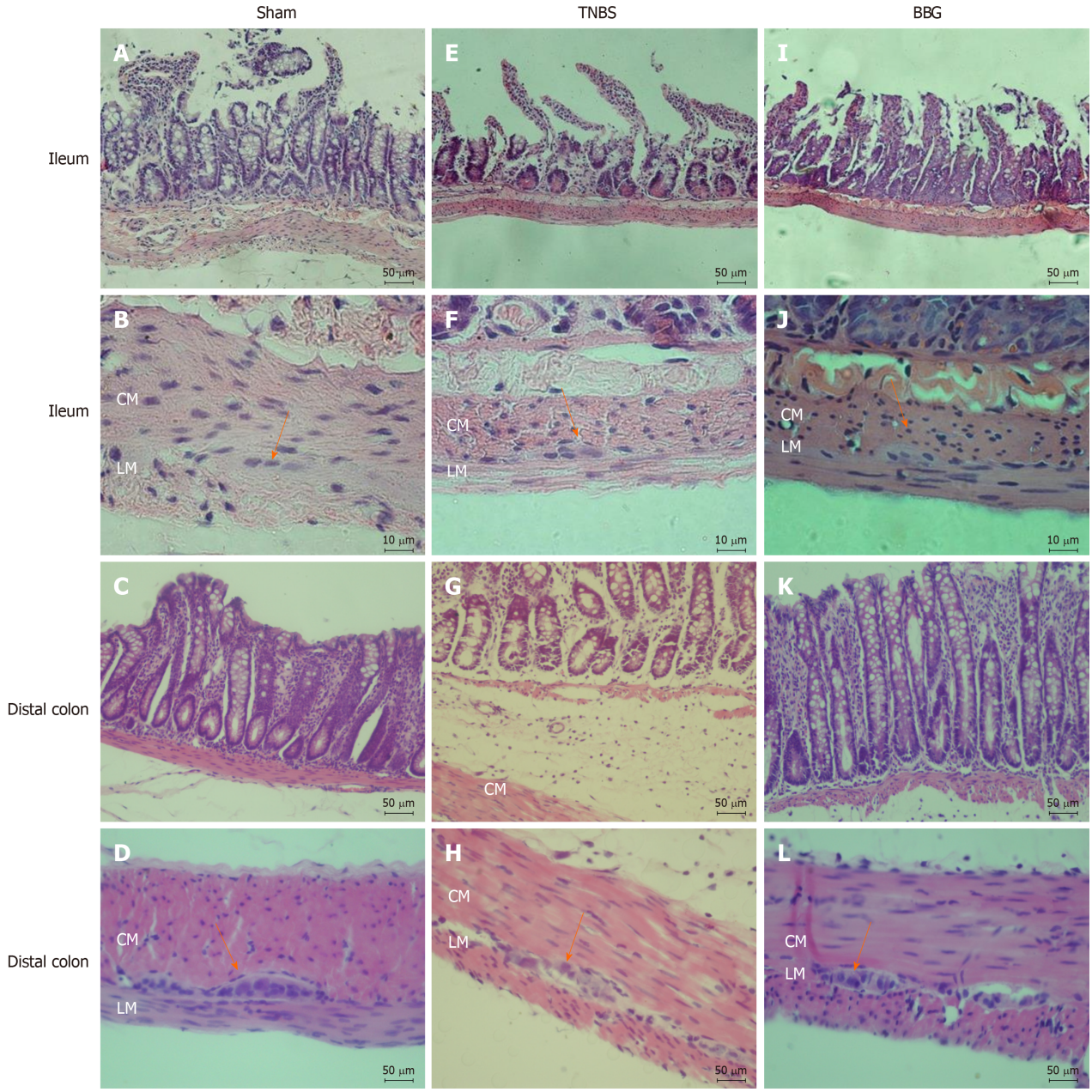

Figure 2 Photomicrographs showing sections stained with hematoxylin and eosin. A, B, E, F, I, J: Rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and brilliant blue G (BBG) groups; C, D, G, H, K, L: Rat distal colon myenteric plexus in the sham, TNBS and BBG groups. The histological observations showed that in ileum the appearances of the mucosa, circular and longitudinal muscles and enteric neurons in the sham, TNBS and BBG groups were preserved. However, the histological observations showed that in distal colon the edema and inflammatory cell infiltration in the TNBS group. The mucosa, the circular and longitudinal muscles and the distal colon enteric neurons in the sham and BBG groups were preserved. Orange arrows indicate myenteric ganglia. CM: Circular muscle; LM: Longitudinal plexus; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G. Scale bars: A, C, D, E, G, H, I, K, L = 50 μm; B, F, J = 10 μm.

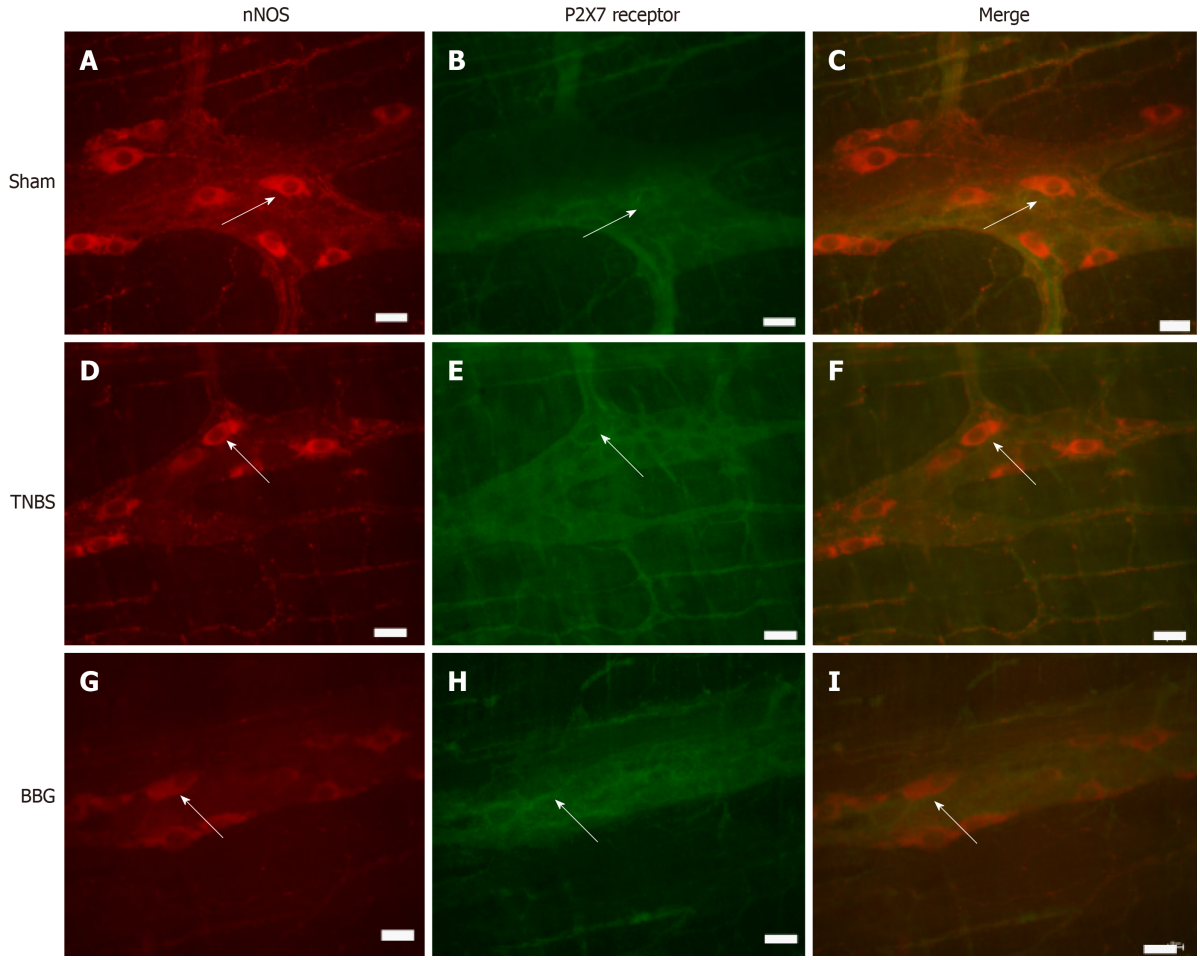

Figure 3 Colocalization of the P2X7 receptor with neuronal nitric oxide synthase in neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. Neuronal nitric oxide synthase immunoreactivity (red; A, D, and G) colocalized with P2X7 immunoreactivity (green; B, E and H). Single arrows indicate double-labeled neurons. Scale bars = 50 μm. nNOS: Neuronal nitric oxide synthase; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

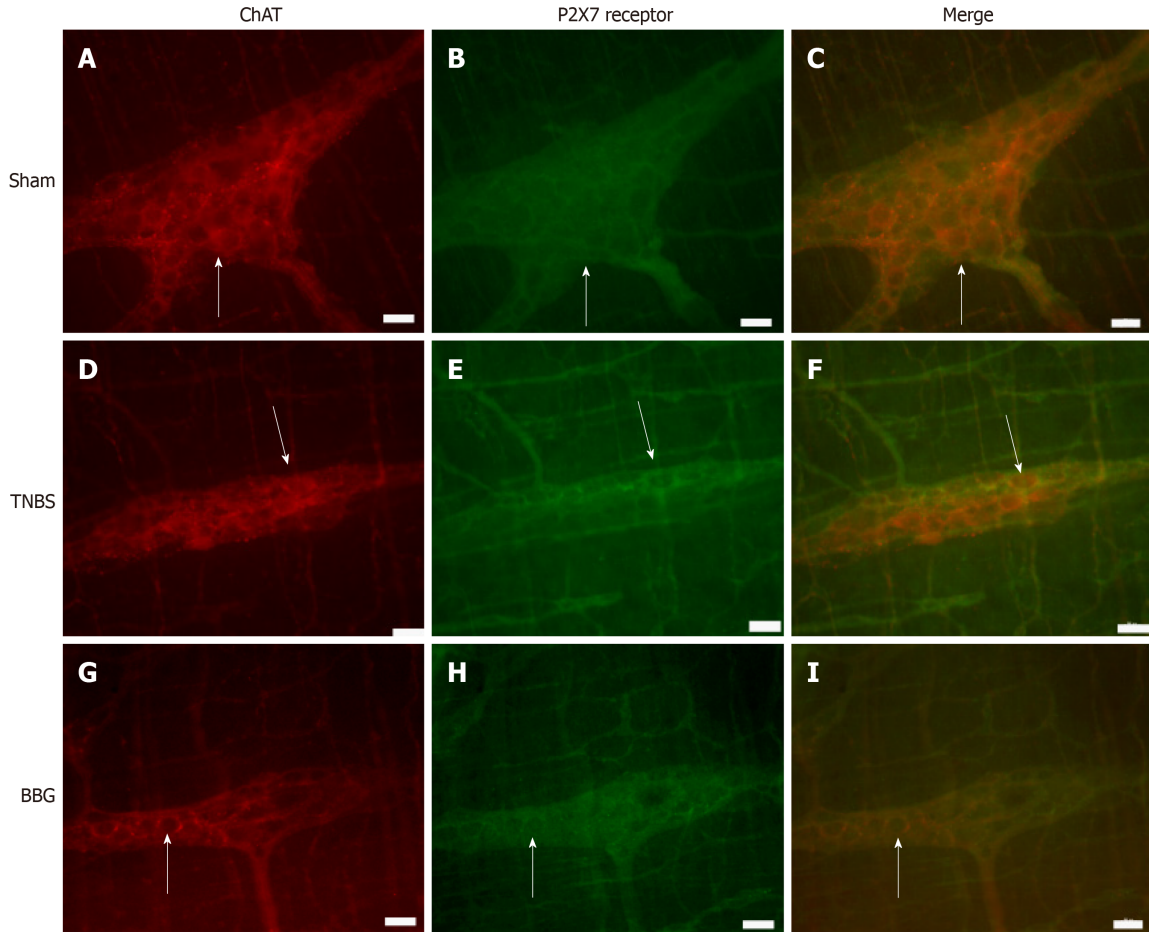

Figure 4 Colocalization of the P2X7 receptor with choline acetyltransferase in neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. Choline acetyltransferase immunoreactivity (red; A, D, and G) colocalized with P2X7 immunoreactivity (green; B, E and H). Single arrows indicate double-labeled neurons. Scale bars = 50 μm. ChAT: Choline acetyltransferase; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

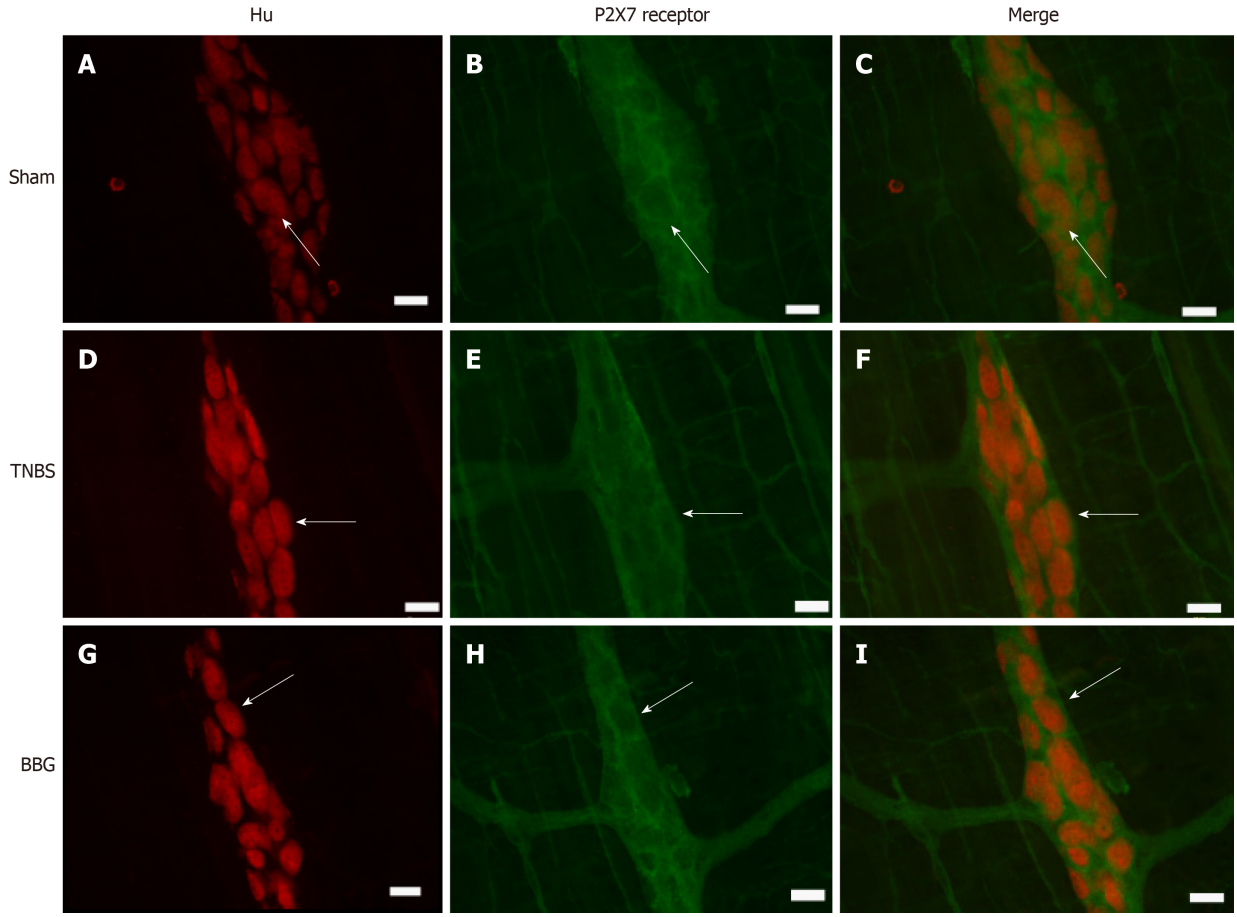

Figure 5 Colocalization of the P2X7 receptor with HuC/D in neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. HuC/D immunoreactivity (red; A, D, and G) colocalized with P2X7 immunoreactivity (green; B, E and H). Single arrows indicate double-labeled neurons. Scale bars = 50 μm. TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

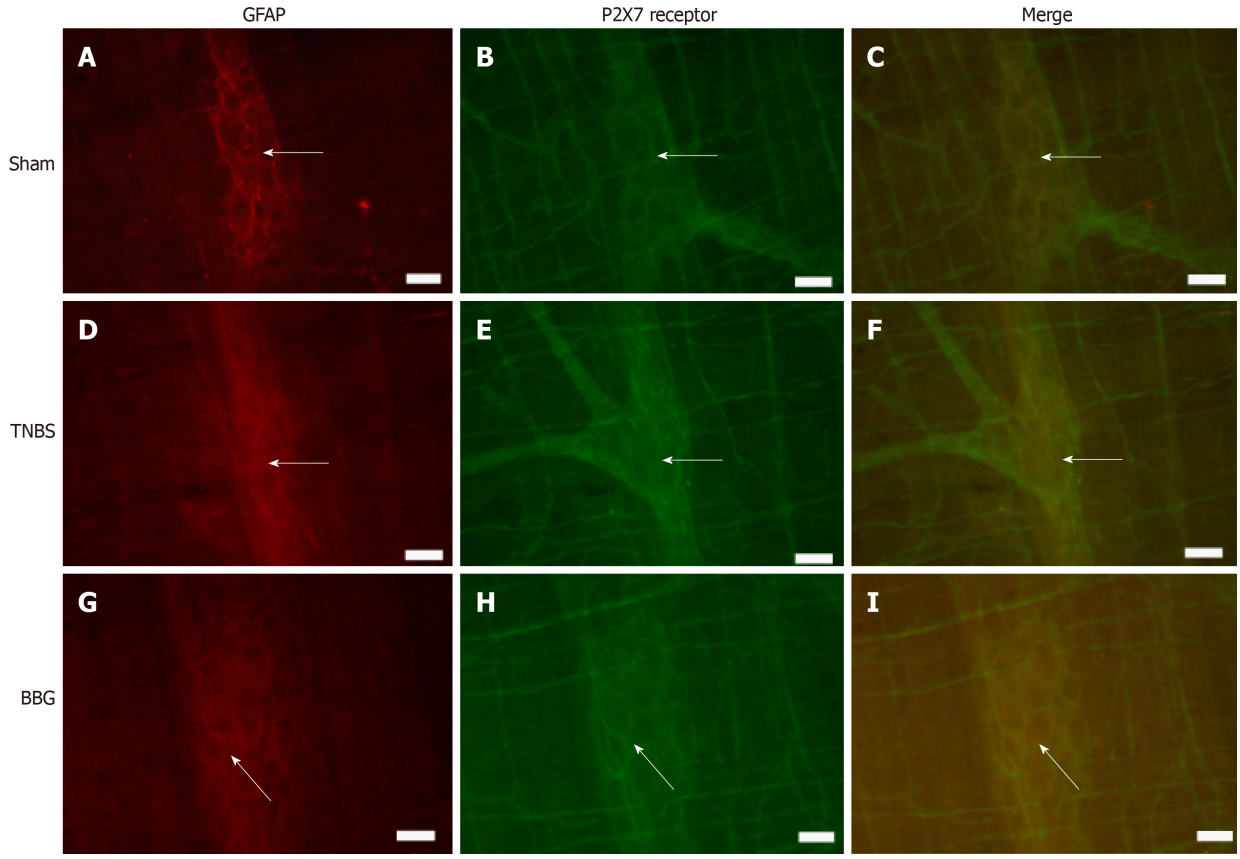

Figure 6 Colocalization of the P2X7 receptor with glial fibrillary acidic protein in the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. GFAP immunoreactivity (red; A, D, and G) colocalized with P2X7 immunoreactivity (green; B, E and H). Single arrows indicate double-labeled enteric glial cells. Scale bars = 50 μm. GFAP: Glial fibrillary acidic protein; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

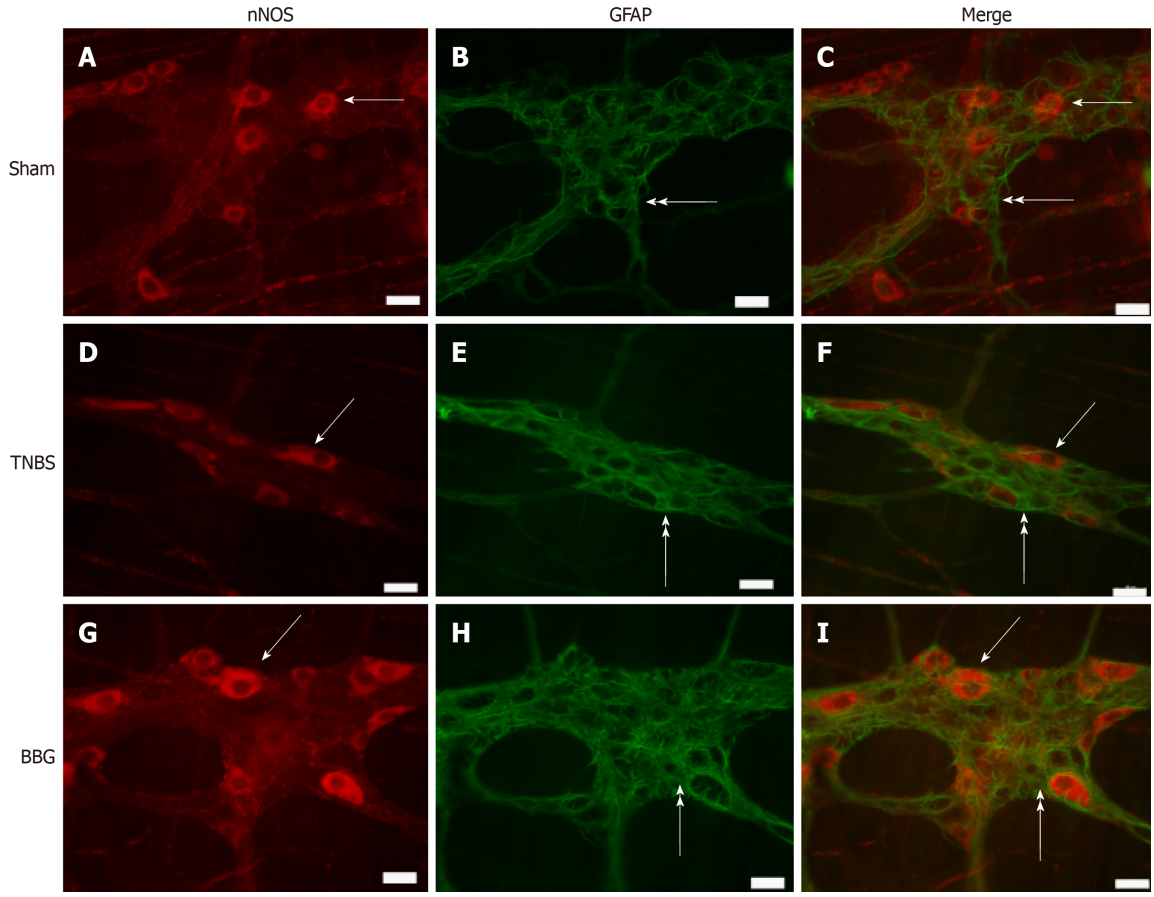

Figure 7 Double labeling of neuronal nitric oxide synthase and glial fibrillary acidic protein in the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. Neuronal nitric oxide synthase immunoreactivity (red; A, D, and G) did not colocalize with glial fibrillary acidic protein immunoreactivity (green; B, E and H). Single arrows indicate labeling of neuronal nitric oxide synthase-positive neurons, and double arrows indicate enteric glial cell positivity. Scale bars = 50 μm. nNOS: Neuronal nitric oxide synthase; GFAP: Glial fibrillary acidic protein; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

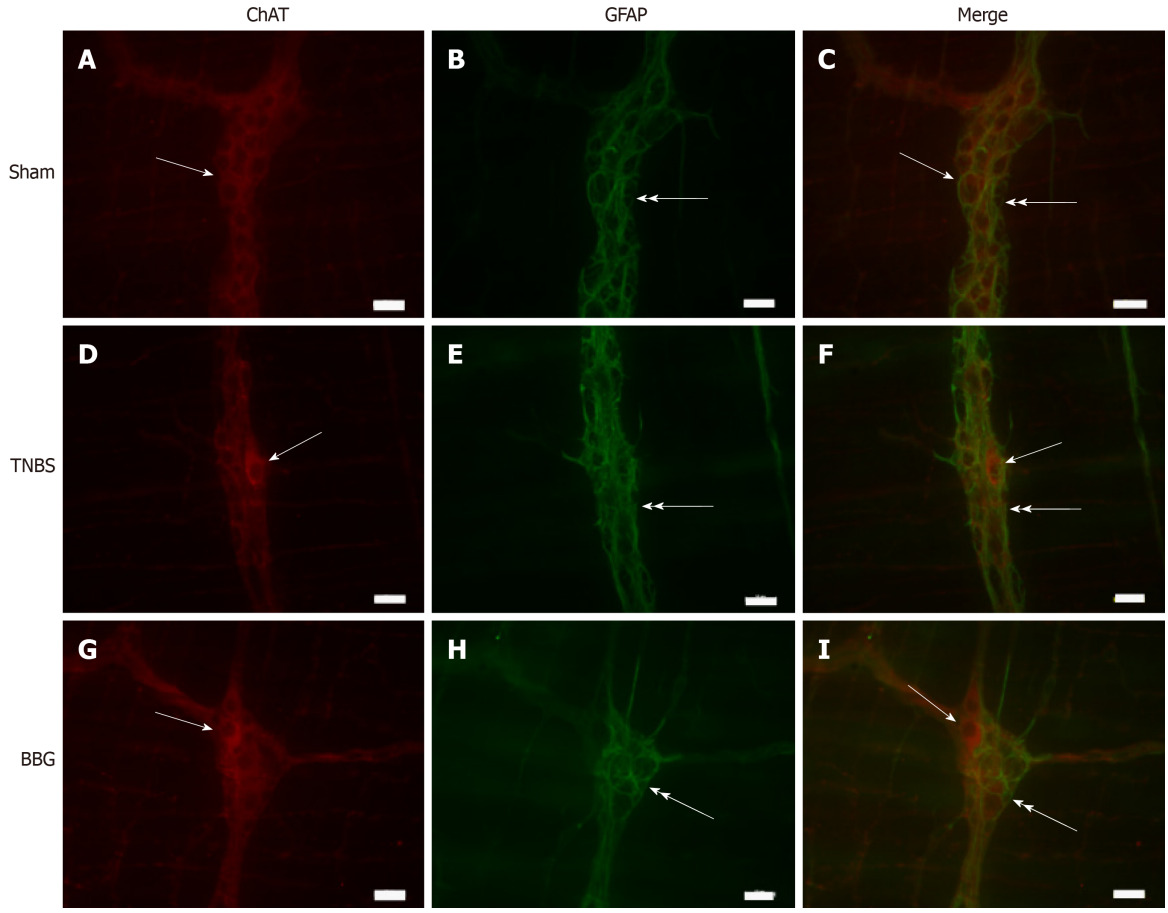

Figure 8 Double labeling of choline acetyltransferase with glial fibrillary acidic protein in the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A-C: Sham group; D-F: 2,4,6-trinitrobenzene group; G-I: Brilliant blue G group. Choline acetyltransferase immunoreactivity (red; A, D, and G) did not colocalize with glial fibrillary acidic protein immunoreactivity (green; B, E and H). Single arrows indicate choline acetyltransferase-positive neurons, and double arrows indicate enteric glial cell positivity. Scale bars = 50 μm. ChAT: Choline acetyltransferase; GFAP: Glial fibrillary acidic protein; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

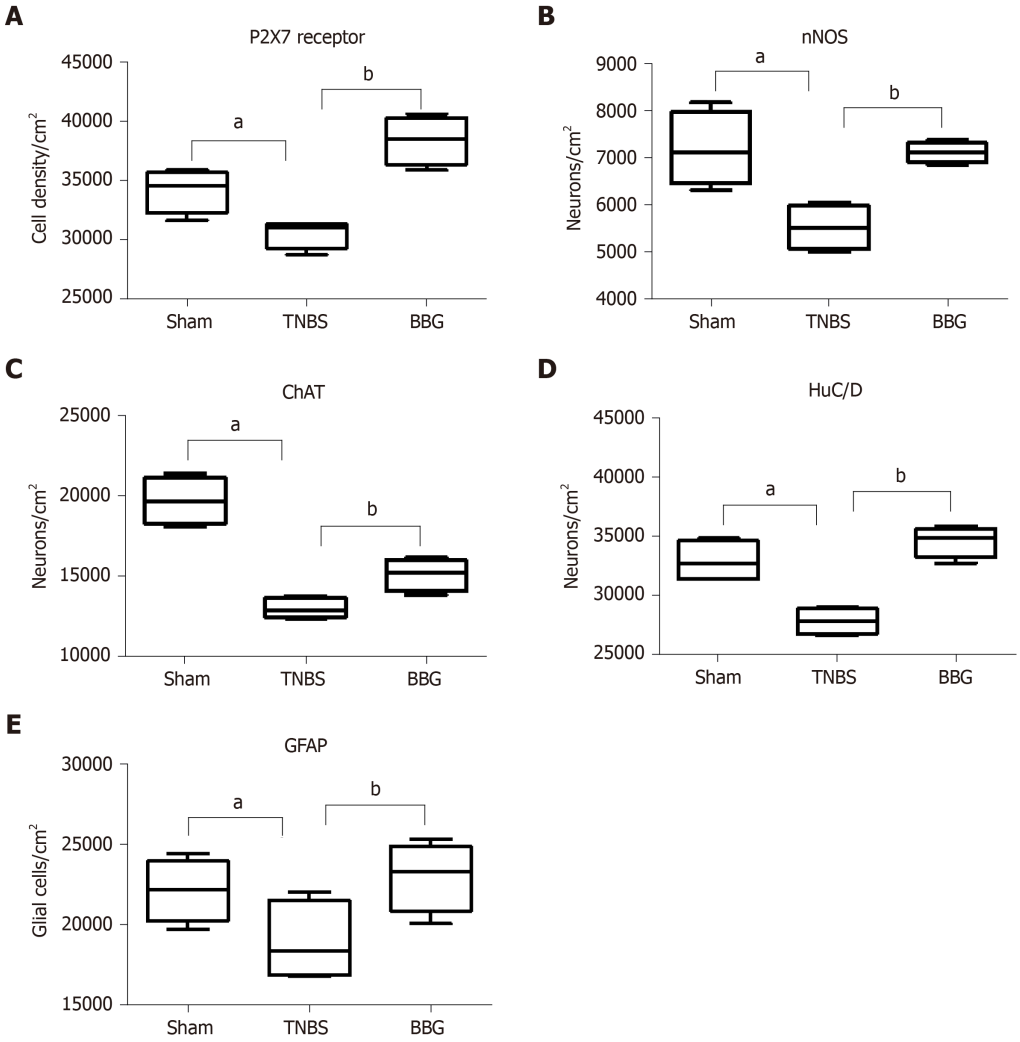

Figure 9 Density of neurons expression in neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A: P2X7 receptor; B: Neuronal nitric oxide synthase; C: Choline acetyltransferase; D: HuC/D; E: Glial fibrillary acidic protein. Counts were made in 40 representative fields for each antigen from each animal from the sham (n = 5), 2,4,6-trinitrobenzene sulfonic acid (TNBS) (n = 5) and brilliant blue G (BBG) groups (n = 5). Data were compared using analysis of variance and Tukey's test for multiple comparisons as appropriate. $P < 0.05$ was considered statistically significant. $^{a}P < 0.05$, comparing the TNBS group and sham group; $^{b}P < 0.05$, comparing the BBG group and TNBS group. The data are expressed as mean ± SE. nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; GFAP: Glial fibrillary acidic protein; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

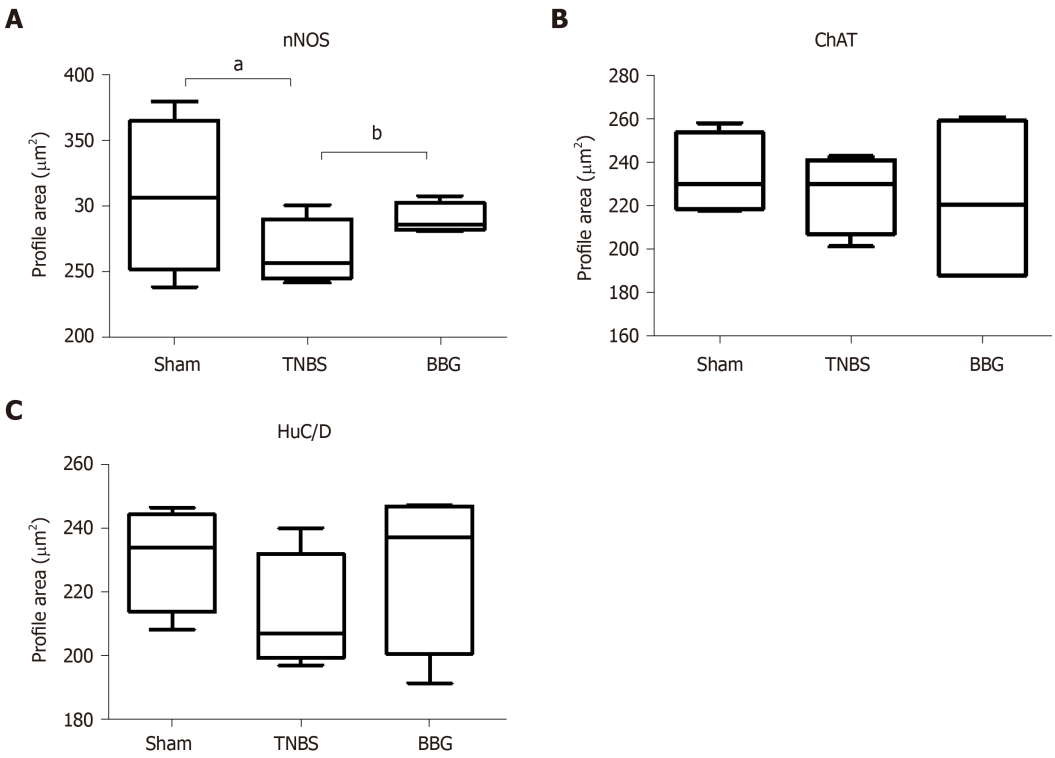

Figure 10 Cell body profile areas of neurons immunoreactive in neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A: Neuronal nitric oxide synthase (nNOS); B: Choline acetyltransferase (ChAT); C: HuC/D. The cell perikaryal profile areas (μm^2) of 100 neurons from each animal were obtained in the sham (n = 5), 2,4,6-trinitrobenzene sulfonic acid (TNBS) (n = 5) and Brilliant blue G (BBG) groups (n = 5). A total of 500 cell profile areas were analyzed for each group. Data were compared using analysis of variance and Tukey's test for multiple comparisons as appropriate. P < 0.05 was considered statistically significant. [a]P < 0.05, comparing the TNBS group and sham group; [b]P < 0.05, comparing the BBG group and TNBS group. The data are expressed as mean ± SE. nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

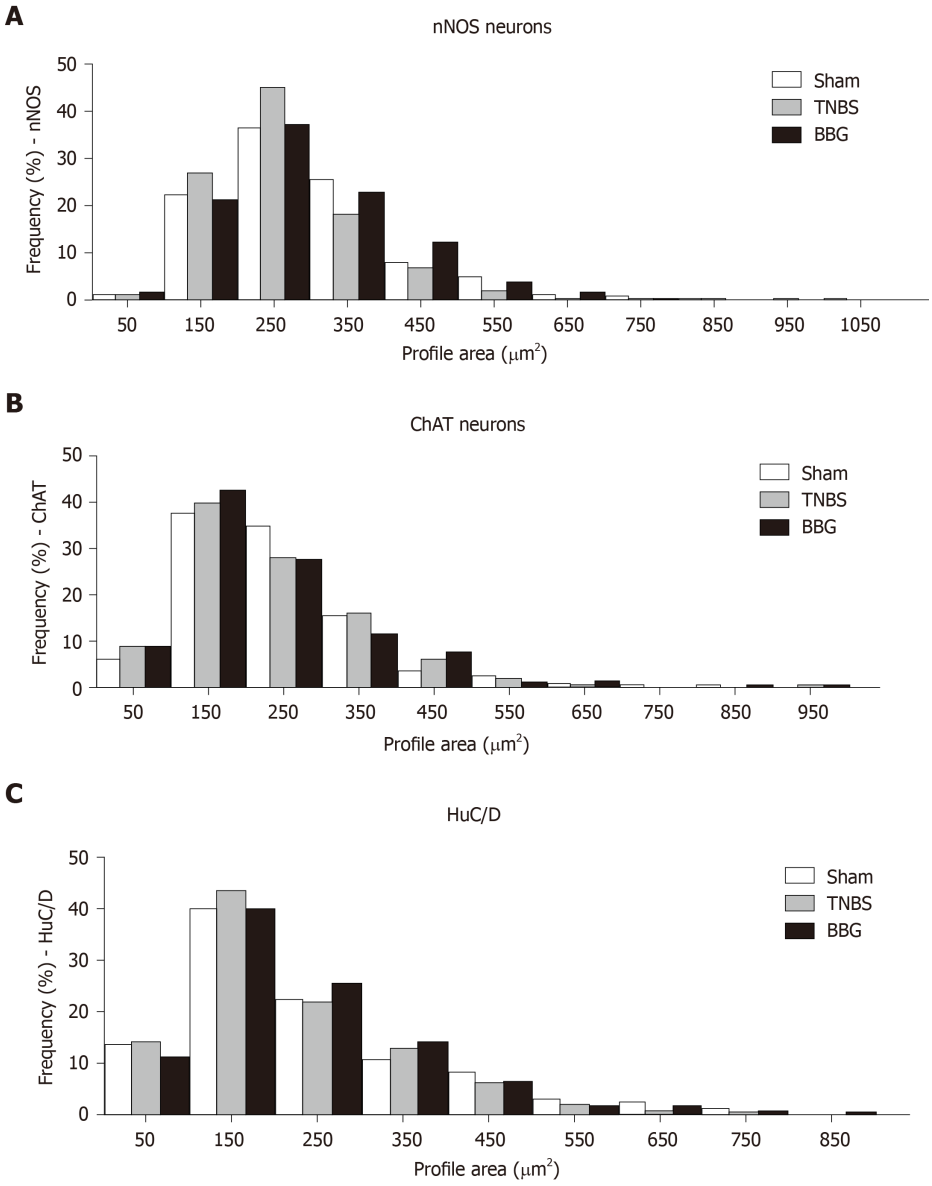

Figure 11 Frequency distribution in cell profiles of neuronal immunoreactivity of neurons among neurons of the rat ileum myenteric plexus in the sham, 2,4,6-trinitrobenzene sulfonic acid and brilliant blue G groups. A: Neuronal nitric oxide synthase (nNOS); B: Choline acetyltransferase (ChAT); C: HuC/D. The size of nNOS-immunoreactive neurons ranged from 50-1050 μm^2. The size of ChAT-immunoreactive neurons ranged from 50-950 μm^2. The size of HuC/D neurons ranged from 50-850 μm^2. The cell perikaryal profile areas of 100 neurons positive for nNOS, ChAT and HuC/D cells from each animal were obtained in the sham (n = 5), 2,4,6-trinitrobenzene sulfonic acid (n = 5) and brilliant blue G groups (n = 5). nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; TNBS: 2,4,6-trinitrobenzene sulfonic acid; BBG: Brilliant blue G.

ARTICLE HIGHLIGHTS

Research background

The enteric nervous system performs functions in gastrointestinal tract such as motility, control of gastric acid secretion, regulation of fluid movement through the epithelium. This system has two ganglionic plexuses, the myenteric plexus and the submucosal plexus. Inflammatory bowel diseases (IBDs) are disorders that include ulcerative colitis and Crohn's disease. In experimental ulcerative colitis, there are changes in enteric neurons. The P2X7 receptor has been described in the ENS.

Research motivation

Studies have demonstrated that P2X7 antagonist, brilliant blue G (BBG) recovers neurons following injuries.

Research objectives

The topics of this work were to analyze the effects of experimental ulcerative colitis in enteric neurons and enteric glial cells in the ileum in animals treated with P2X7 antagonist (BBG).

Research methods
The rats were anesthetized with a mixture of xylazine (20 mg/kg) and ketamine (100 mg/kg) administered subcutaneously. Inflammation was induced through the intrarectal insertion of a polypropylene 8 cm cannula. 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma, Saint Louis, United States) was injected at a dose of 30 mg/kg in 600 µL of 30% ethanol in the colon lumen (n = 5). Sham animals (n = 5) were injected with vehicle. BBG (50 mg/kg, Sigma Aldrich, United Kingdom, n = 5) or saline was injected 1 h following TNBS injection (n = 5). The survival time after colitis induction was 24 h. For immunohistochemistry, fresh segments of the ileum were dissected after fixed. Double labeling has been done of P2X7 receptor with neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), and HuC/D (a pan-neuronal marker) and enteric glial cells immunoreactive for glial fibrillary acidic protein (GFAP). The stained tissue specimens were examined using a Nikon 80i fluorescent and Confocal microscope. The counting of the neurons per area and glial cell were done in fluorescent microscope.

Research results
The numbers of nNOS-, ChAT-, HuC/D- immunoreactive (ir) neurons and GFAP-ir glial cells were decreased in the TNBS group and recovered in the BBG group. The neuronal profile area (μm^2) demonstrated that nNOS-ir neurons decreased in the TNBS group and recovered in the BBG group. There were no differences in the profile areas of ChAT- and HuC/D-ir neurons. Our data conclude that ileum myenteric neurons and glial cells were affected by ulcerative colitis and that treatment with BBG had a neuroprotective effect. Thus, these results demonstrate that the P2X7 receptor may be an important target in therapeutic strategies.

Research conclusions
Ileum myenteric neurons and glial cells were affected by experimental ulcerative colitis and that treatment with P2X7 receptor antagonist, BBG had a neuroprotective effect. The results demonstrate that the P2X7 receptor may be an important target in therapeutic strategies. P2X7 receptor may be a possible therapeutic target in the treatment of the effects of experimental ulcerative colitis Ileum myenteric neurons and glial cells were affected by experimental ulcerative colitis and treatment with BBG may recover enteric neurons. P2X7 receptor may be a possible therapeutic target in the treatment of the experimental ulcerative colitis. Injection of BBG (50 mg/kg, Sigma Aldrich, United Kingdom) for experimental ulcerative colitis and effects in the distal ileum. Inflammation was induced through the intrarectal insertion of a polypropylene 8 cm cannula. 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma, Saint Louis, United States) was injected at a dose of 30 mg/kg in 600 µL of 30% ethanol in the colon lumen. There was affected the distal ileum. Additionally, injection of BBG recover enteric neurons distal ileum. Studies show that BBG is a P2X7 antagonist, and its low toxicity and high selectivity make this compound an ideal candidate to block the adverse effects of P2X7 receptor activation. BBG treatment was shown to be effective in the recovery of ileum myenteric neurons, thus demonstrating that the P2X7 receptor may be a possible therapeutic target in the treatment of the effects of experimental ulcerative colitis.

Research perspectives
Study of effects of the experimental ulcerative colitis in the ileum and may use of the P2X7 receptor for therapeutic target. Additionally, study effects of BBG in the distal colon following experimental ulcerative colitis. The direction of the future research will be study effects of the experimental ulcerative colitis of myenteric neurons in the P2X7 receptor-deficient animals. The best method will be use P2X7 receptor-deficient animals.

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OPINION REVIEW

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ABOUT COVER

Editorial board member of *World Journal of Gastrointestinal Pathophysiology*, Dr. Yang is a Professor in the Third Affiliated Hospital, the Chinese University of Hong Kong (Shenzhen), Shenzhen. He became an Associate Professor of Northwest Normal University in 2007 and was promoted to Professor in Lanzhou Institute of Chemical Physics, CAS in 2009. His ongoing research interests are colorectal laterally spreading tumors (LSTs), gastric carcinoma and coronavirus infectious disease. He is a member of the editorial boards and a reviewer of several scientific journals, and he has published more than 100 peer-reviewed articles, including NEJM, Carbon, Virology, and World Journal Gastroenterology, and so on. Currently, he is the PI of the Research Center of Gastroenterology in the Third Affiliated Hospital, the Chinese University of Hong Kong (Shenzhen).

AIMS AND SCOPE

The primary aim of the *World Journal of Gastrointestinal Pathophysiology* (*WJGP*, *World J Gastrointest Pathophysiol*) is to provide scholars and readers from various fields of gastrointestinal pathophysiology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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OPINION REVIEW

Atherosclerotic cardiovascular disease in inflammatory bowel disease: The role of chronic inflammation

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Abstract

Inflammatory bowel disease (IBD) causes systemic vascular inflammation. The increased risk of venous as well as arterial thromboembolic phenomena in IBD is well established. More recently, a relationship between IBD and atherosclerotic cardiovascular disease (ASCVD) has been postulated. Systemic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus, have well characterized cardiac pathologies and treatments that focus on prevention of disease associated ASCVD. The impact of chronic inflammation on ASCVD in IBD remains poorly characterized. This manuscript aims to review and summarize the current literature pertaining to IBD and ASCVD with respect to its

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pathophysiology and impact of medications in order to encourage further research that can improve understanding and help develop clinical recommendations for prevention and management of ASCVD in patients with IBD.

Key words: Crohn's disease; Ulcerative colitis; Atherosclerosis; Thromboembolism; Chronic inflammation; Pathophysiology

Core tip: Chronic inflammation in patients with inflammatory bowel disease (IBD) leads to increased risk of atherosclerotic cardiovascular disease (ASCVD). However, the role and potential impact of IBD therapy in modifying ASCVD risk, and vice-versa, remains poorly understood. Herein, we highlight the importance of ASCVD as an extraintestinal manifestation of IBD, discuss the pathophysiology common to both diseases, and explore the role of non-traditional risk factors of ASCVD in IBD. We intend to identify avenues for further clinical and translational research that may help develop clinical recommendations for the management of ASCVD risk in patients with IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, systemic, relapsing and remitting inflammatory disorder of the gut due to immune and endothelial dysfunction in genetically susceptible hosts[1]. The two main phenotypic patterns of IBD are Crohn's disease (CD) and ulcerative colitis (UC)[2]. In addition to the primary gastrointestinal manifestations of IBD, a wide-range of extra-intestinal manifestations (EIMs) have also been reported[3,4]. One of the well-recognized EIMs of IBD is the increased risk of thromboembolic phenomena[5-7]. Several recent studies have demonstrated a two to four-fold increased risk of atherosclerotic cardiovascular disease (ASCVD) in patients with IBD as compared to the general population[8]. There is growing evidence that atherosclerosis involves dysregulation of innate and adaptive immune systems along with platelet and endothelial dysfunction. Hence, it is believed that the underlying chronic inflammatory process in patients with IBD, similar to other chronic inflammatory disorders, may drive ASCVD risk[9,10]. The goal of this manuscript is to review the association between ASCVD and IBD as well as identify avenues for further research that may help develop clinical recommendations for the management of ASCVD risk in patients with IBD.

ASCVD AS AN EIM OF IBD

Patients with IBD are exposed to chronic, persistent, systemic inflammation over the course of their lifespan. This predisposes them to a wide range of sequelae across multiple organ systems. The EIMs associated with IBD are traditionally classified as primary and secondary[11]. Primary EIMs include joint, orocutaneous, ophthalmological, hepatobiliary, pulmonary, and renal manifestations (Figure 1). Among these, some parallel IBD disease activity (episcleritis, pyoderma gangrenosum, erythema nodosum, aphthous stomatitis, peripheral arthropathy) and others do not (uveitis, ankylosing spondylitis, primary biliary cirrhosis, alopecia areata, thyroid autoimmune disease). Secondary EIMs are complications related to IBD and include osteoporosis, anemia, cholelithiasis, nephrolithiasis, and thromboembolism (Figure 1)[10].

The relationship between IBD and venous thromboembolic phenomena has been

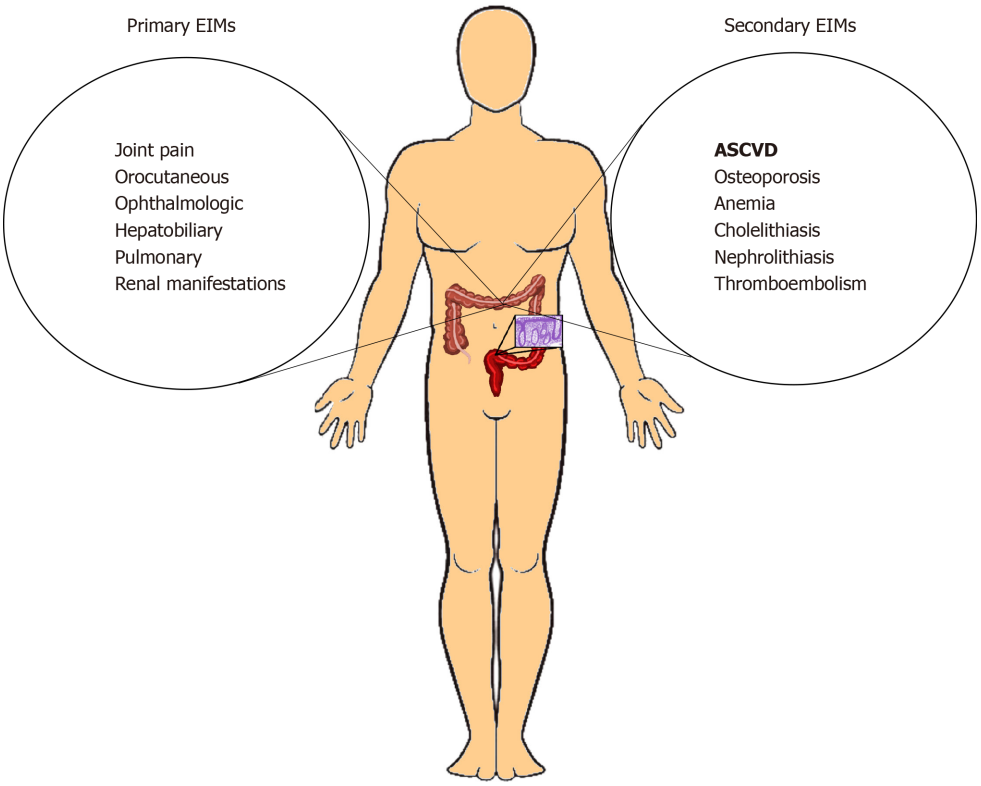

Figure 1 Illustration summarizing the primary and secondary extra-intestinal manifestations associated with inflammatory bowel disease, including atherosclerotic cardiovascular disease as a more recently recognized secondary extra-intestinal manifestation of inflammatory bowel disease. EIM: Extra-intestinal manifestation; ASCVD: Atherosclerotic cardiovascular disease.

well characterized[12-14]. Patients with IBD have a 1.7 to 5.9 times increased risk of venous thromboembolism as compared to the general population[14,15,16]. The underlying pathophysiologic mechanism of thromboembolic phenomena in IBD merits further investigation.

Labeling ASCVD as an EIM of IBD adds valuable understanding to the role of chronic inflammation in the two disease processes. Currently, we do not have enough knowledge regarding the effect of decreasing IBD disease burden on ASCVD and vice versa to categorize it as primary versus secondary EIM. There are no clinical human trials and long-term prospective data to assess whether ASCVD will follow the trajectory of gut inflammation in patients with IBD. Whether there is a specific time point in vascular disease after which reversibility is unachievable needs to be evaluated. The answer to these questions will help us more appropriately place ASCVD in the subcategories of EIM related to IBD. With current knowledge it can be categorized as a secondary EIM (Figure 1).

ASCVD AND IBD

The incidence of ASCVD is increased in patients with IBD as reported by multiple population-based and few retrospective case control studies[17-22]. Indeed, there is a two to four-fold increased risk of myocardial infarction (MI), stroke and heart failure (HF) in patients with IBD. Data from Danish and other European cohort studies, for example have shown an association between ischemic heart disease (IHD) and IBD[17,20-22]. A Canadian study reported an increased risk of IHD (IRR 1.26; 95%CI: 1.11-1.44) and cerebrovascular accidents in patients with IBD (IRR 1.32; 95%CI: 1.05–1.66)[22]. Panhwar *et al*[8] analyzed inpatient and outpatient data from the Unites States and reported a two-fold increased risk of MI in patients with IBD[8].

Some studies and meta-analyses, however, have shown conflicting data[23,24]. Several reasons may account for this; for example, large retrospective cohort studies rely on billing data that are prone to miscoding. In addition, many studies lack data on disease activity and medication use that can influence the inflammatory process and hence the ASCVD events and risk. Studies that have assessed disease activity through indirect

measures like steroid use or inpatient admissions have shown a more consistent positive association between the two inflammatory processes[19,20]. A pattern that has emerged over the years shows an increased risk of ASCVD with active disease and in younger and female patients as compared to the general population[9,20]. Notably, there are data showing increased subclinical atherosclerosis in patients with IBD as assessed by modalities such as carotid femoral pulse wave velocity, carotid intimal thickening, and flow-mediated dilatation (FMD)[24,25].

The effect of ASCVD related mortality in patients with IBD is debatable[12,23,26-29]. This can be attributed to multiple factors. Some studies assess mortality over the short duration of inpatient admission that might undermine the effect of long standing chronic inflammation[23,29,30]. Given the increased overall mortality among patients with IBD, it is possible that survivorship and participation bias are obscuring the potential IBD-ASCVD relationship[16]. Some studies lack rigorous matching of the control population[30]. Prospective studies with age matched control groups evaluating the role of IBD medications may be able to better assess the relationship between IBD and ASCVD.

NON-TRADITIONAL RISK FACTORS OF ASCVD IN PATIENTS WITH IBD

Traditional risk factors for ASCVD are age (men > 45 years, women > 55 years), male gender, family history of coronary artery disease (CAD), obesity [body mass index (BMI) > 30], hypertension, diabetes, dyslipidemia, tobacco use, alcohol use, and chronic kidney disease. However, patients with IBD tend to have lower BMI and no significant lipid abnormality[18]. Aspects like age and gender have shown deviation from the general population. Nontraditional risk factors of arterial thromboembolism in patients with IBD merit further investigation.

Role of inflammatory markers

IBD is characterized by high levels of C-reactive protein and various biomarkers that are associated with ASCVD, such as oxidized-low density lipoprotein, fibrinogen, matrix metallopeptidase, nuclear factor kappa-B, and interferon-γ. These are known to cause endothelial dysfunction, platelet aggregation, and hasten the development of ASCVD[31].

In a retrospective case control study, 356 IBD and 712 matched control patients, were assessed with an average follow up time of four and a half years. An increased incidence of ASCVD in patients with IBD despite having a lower burden of traditional risk factors was reported[18]. Furthermore, some non-traditional risk factors, such as white blood cells or perhaps even chronic inflammation, may have had a more robust impact on ASCVD in patients with IBD as compared to the traditional risk factors of hypertension, obesity, and hyperlipidemia[18]. We discuss in this manuscript (pathophysiology section) the biomarkers that link the two disease processes and have the potential for further translational research.

Increased ASCVD with disease activity

A higher risk of venous thromboembolism during active disease is well studied[9] Even though some studies have shown contrary results regarding the relationship between ASCVD and IBD, a consistent pattern of increased incidence of MI during periods of active disease has been evident[12,23,32,33]. A Danish cohort study evaluated 20795 patients with IBD and 199978 matched controls and showed increased risk of MI, stroke, and cardiovascular death during periods of active IBD. During periods of remission, these risks were similar to controls[17]. A French cohort study assessed MI, stroke and peripheral vascular disease in patients 30 days before and after hospital admission, which was taken as a surrogate marker for IBD flare, and found an increased risk of arterial thromboembolic events suggesting the importance of ongoing inflammation and its affect beyond the length of hospital admission[20]. A recent study from Olmsted County, Minnesota, showed an increased risk of MI and HF in patients with IBD with more active and extensive disease[19].

There have been no prospective studies to assess disease activity by stool, blood or clinical markers and correlate it with ASCVD events over short-term or long-term. Most studies have used surrogate markers of inflammation like steroid use, hospitalization or escalation of therapy for disease activity[34]. Studies that measure disease activity and its duration in a prospective manner are needed to better assess the outcomes of ASCVD in patients with IBD.

Increased ASCVD risk in female and younger patients with IBD as compared to the general population

Multiple studies have shown that ASCVD tends to occur in women and younger patients with IBD as compared to the general population[8,35-39]. In a meta-analysis by Sun *et al*, data from 27 studies showed pooled relative risk for ASCVD, CAD, and MI was 1.25 (95%CI: 1.08-1.44), 1.17 (95%CI: 1.07-1.27) and 1.12 (95%CI: 1.05-1.21), respectively. An association was particularly noted in women with IBD[40]. The meta-analysis by Singh *et al*[40] identified an overall 19% increased risk of IHD among patients with IBD, however subgroup analysis found the risk among women with IBD to be 26% and the risk among men fell below the level of statistical significance. A Danish population based study that showed an increased risk of IHD in patients with IBD (IRR 1.22, 95%CI: 1.14-1.30) noted that the risk was higher in women (IRR 1.33, 95%CI: 1.21- 1.46) and younger patients of age 15-34 years (IRR 1.37, 95%CI: 0.98-1.93)[37]. An Asian cohort study showed that the adjusted hazard ratio for acute coronary syndrome in patients with IBD, compared with controls, was highest in 20 to 39 year age group at 3.28 (95%CI: 1.73-6.22), as compared to the overall risk of 1.70 (95%CI: 1.45-1.99)[36]. Recently published study from a large United States database reported higher prevalence of IHD in patients with UC and CD as compared to non-IBD patients [UC 6.9% *vs* CD 9.0% *vs* non-IBD 4.0%, OR for UC 2.09 (2.04-2.13), and CD 2.79 (2.74–2.85)]. In this patient cohort, the prevalence of MI was highest among younger patients with IBD[8].

It has been shown that patients with other chronic inflammatory conditions, *e.g.*, psoriasis, also have evidence of ASCVD at a younger age than general population[38]. Younger CD patients have more aggressive disease with more disease burden early during their life and hence it is important to assess their risk of other pro-inflammatory conditions like atherosclerosis[35]. Investigating and identifying the factors that influence this increased risk (*e.g.*, disease duration and severity) in patients with IBD will help start early intervention with appropriate therapy and thereby decrease inflammatory burden, aimed at long-term risk modification.

PATHOPHYSIOLOGY LINKING THE TWO DISEASES

Atherosclerosis is the most common cause of ischemic cardiomyopathy and vascular disease. Chronic inflammation involving the innate and adaptive immune system along with endothelial and platelet dysfunction is present in both atherosclerosis and IBD.

Endothelial cells, lymphocytes, monocytes, macrophages are all involved in the pathogenesis of atherosclerosis from formation of foam cells to development of plaque[39,41-44]. Disruption of the endothelium early in the process of atherosclerosis leads to upregulation of adhesion molecules, deposition of lipoproteins in the subendothelial and recruitment of circulating monocytes from the spleen and bone marrow. Some of the adhesions molecules like VCAM1, P-selectin, and ICAM1 are notable in this pathway that lead to expression of chemokines like CCR1, CCR5 and CX3C receptor 1[45-48]. Many of these adhesions' molecules have been implicated in the pathogenesis of IBD and as potential therapeutic drug targets. Vedolizumab is an anti-integrin molecule that prevents recruitment of white blood cells to the gut by inhibiting binding of $\alpha_4\beta_7$ adhesion molecules on the monocytes to the endothelial cells and is widely used to treat CD and UC[49]. Exploring the effect of such medications on ASCVD risk in IBD patients is a potential avenue of research. Further in the process of atherosclerosis, the recruited monocytes differentiate into activated macrophages and take up the apoB containing lipoproteins leading to lipid accumulation and formation of macrophage driven foam cells that overtime lay the foundation of a necrotic lipid core[50-52]. The role of endothelial dysfunction in patients with IBD has been evaluated in a small study through nitric oxide mediated dilatation of the vessels[41]. Endothelial dysfunction as assessed by FMD has been shown to be impaired in patients with active UC[42].

In addition to the innate immune system (macrophages) and endothelial dysfunction (adhesion molecules), the adaptive immune system (T and B lymphocytes, dendritic cells) is involved in the pathogenesis of atherosclerosis. Activation of T helper 1(T_H) leads to production of pro-inflammatory cytokines [interleukin (IL)-1, IL-6, tumor necrosis factor] that activate local inflammatory cascade[53]. These cytokines are also involved in the pathogenesis of IBD through activation of T_H1 and T_H17 cells in CD and T_H2 cells in UC. The JAK and STAT pathways that act downstream of cytokine mediated lymphocyte activation are implicated in the activation of IL-6 in

atherosclerosis and IBD[54]. The JAK inhibitors are being extensively explored as a therapeutic target in IBD and tofacitinib (JAK 1/3 inhibitor) is currently used for treatment of moderate to severe UC[55]. Infliximab and other anti TNF medications are known therapeutic targets in IBD[56,57].

Translational research assessing pathways and cytokines of innate and adaptive immune system and, vascular endothelial dysfunction that are common to both inflammatory processes will help identify biomarkers that can be used to assess, risk stratify and develop focused preventive and treatment modalities to reduce ASCVD risk in patients with IBD.

ASCVD RISK MODIFICATION FROM ANTI-INFLAMMATORY MEDICATIONS

Numerous studies have examined an overlap in treatment between IBD and heart disease, with relative success (Table 1). In a retrospective matched case–control study Ungaro *et al*[43] assessed statin use in patients with IBD. Statin use was associated with a significantly decreased risk of IBD (OR: 0.68, 95%CI: 0.64-0.72), CD (OR: 0.64, 95%CI: 0.59-0.71), and UC (OR: 0.70, 95%CI: 0.65-0.76)[43]. Two studies developed animal models of IBD by chemically inducing CD and UC and showed that captopril was implicated in reducing transforming growth factor-beta1 expression and colitis-associated fibrosis[44,58]. Clopidogrel also reduced disease activity index and colonic mucosal damage index in mice – thus providing an additional link between treatment of IBD and ASCVD[58].

In addition to studies showing benefit for patients with IBD taking ASCVD medications, there have been studies assessing the role of anti-inflammatory medications for ASCVD in the general population. In a multicenter randomized control (CANTOS) trial by Ridker *et al*[59], canakinumab, a human monoclonal antibody targeted at IL-1, showed a 15% reduction in deaths from heart attacks and strokes combined. Further analysis of the CANTOS trial demonstrated that patients receiving canakinumab that achieved IL-6 levels below 1.65 ng/L had a 32% reduction in major ASCVD events, whereas those at or above 1.65 ng/L received no ASCVD-related benefits[54]. Although there was an increase in fatal infections in the treatment group resulting in no overall mortality benefit, it was clearly illustrated that treatment of inflammation independent of lipid levels resulted in fewer cardiovascular events, and IL-6 could be a potential common mechanism. In addition, a nationwide retrospective cohort study noted that for patients with IBD taking 5-aminosalicylic acid (5-ASA), the incidence of IHD was significantly less compared to those not taking 5-ASA[37]. A meta-analysis assessing anti-TNF use in IBD has given us some direction regarding medications that can influence disease activity and ASCVD in patients with IBD[60]. A multicenter prospective longitudinal study in patients with IBD evaluated arterial pulse wave velocity (a surrogate marker of subclinical atherosclerosis) found improvement with long-term anti–TNF therapy suggesting that reduction of inflammation can lead to improvement in endothelial dysfunction[61].

CONCLUSION

Clinicians should be aware of the harmful effects of chronic inflammation on the heart. ASCVD risk is increased among patients with IBD especially during periods of active disease. ASCVD in patients with IBD tends to favor non-traditional risk factors like younger age and female gender. The role of inflammatory markers of IBD as risk factors for ASCVD needs further investigation. Appropriate risk stratification is important in all age groups but especially in those that are diagnosed at an early age and carry the disease burden over a long time. Early escalation of care with more aggressive anti-inflammatory therapy may have a beneficial effect on chronic inflammatory processes like ASCVD, but this needs to be evaluated. Prospective studies assessing the role of IBD medications on ASCVD risk and events will help tailor therapy in patients with IBD based on the mechanism of the drug and subsequently help us move towards personalized medicine.

Table 1 Clinical studies that have found overlap in treatment between inflammatory bowel disease and atherosclerotic cardiovascular disease indicating that the underlying chronic inflammatory process in patients with inflammatory bowel disease may drive atherosclerotic cardiovascular disease risk, and vice versa

Ref.	Study type	The number of patients	Efficacy in treatment overlap
Ungaro *et al*[43]	Retrospective matched case-control study	A total of 9617 cases and 46665 controls were included in the analysis	Statistically significant decreased risk of new onset IBD in patients taking statin therapy
Wengrower *et al*[44]	Chemically induced colitis, animal model	A total of 40 male rats were divided into normal control, captopril only, induced-colitis control, and induced-colitis with captopril arms	A significant reduction in fibrosis, levels of TGF-beta1 mRNA, and protein was found in patients with IBD taking captopril
Patel *et al*[58]	Chemically induced Crohn's and UC, animal model	A total of 48 mice, divided into induced Crohn's and UC arms, further divided into normal, disease, disease with standard therapy, and disease with clopidogrel	A statistically significant reduction in disease activity and colonic mucosal damage was seen in mice on clopidogrel
Rungoe *et al*[57]	Nationwide, population-based retrospective cohort study	A total of 28833 patients with IBD were compared to matched non-IBD patients from a dataset of 4.6 million	A statistically significant reduction in risk of ischemic heart disease was seen in patients given 5-ASA
Zanoli *et al*[61]	Multicenter prospective longitudinal study	334 patients with IBD were followed for 4 yr	In patients with IBD anti-TNFα therapy reduced aortic pulse-wave velocity (a surrogate for cardiovascular risk)

IBD: Inflammatory bowel disease; TGF: Transforming growth factor; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylic acid; TNF: Tumor necrosis factor.

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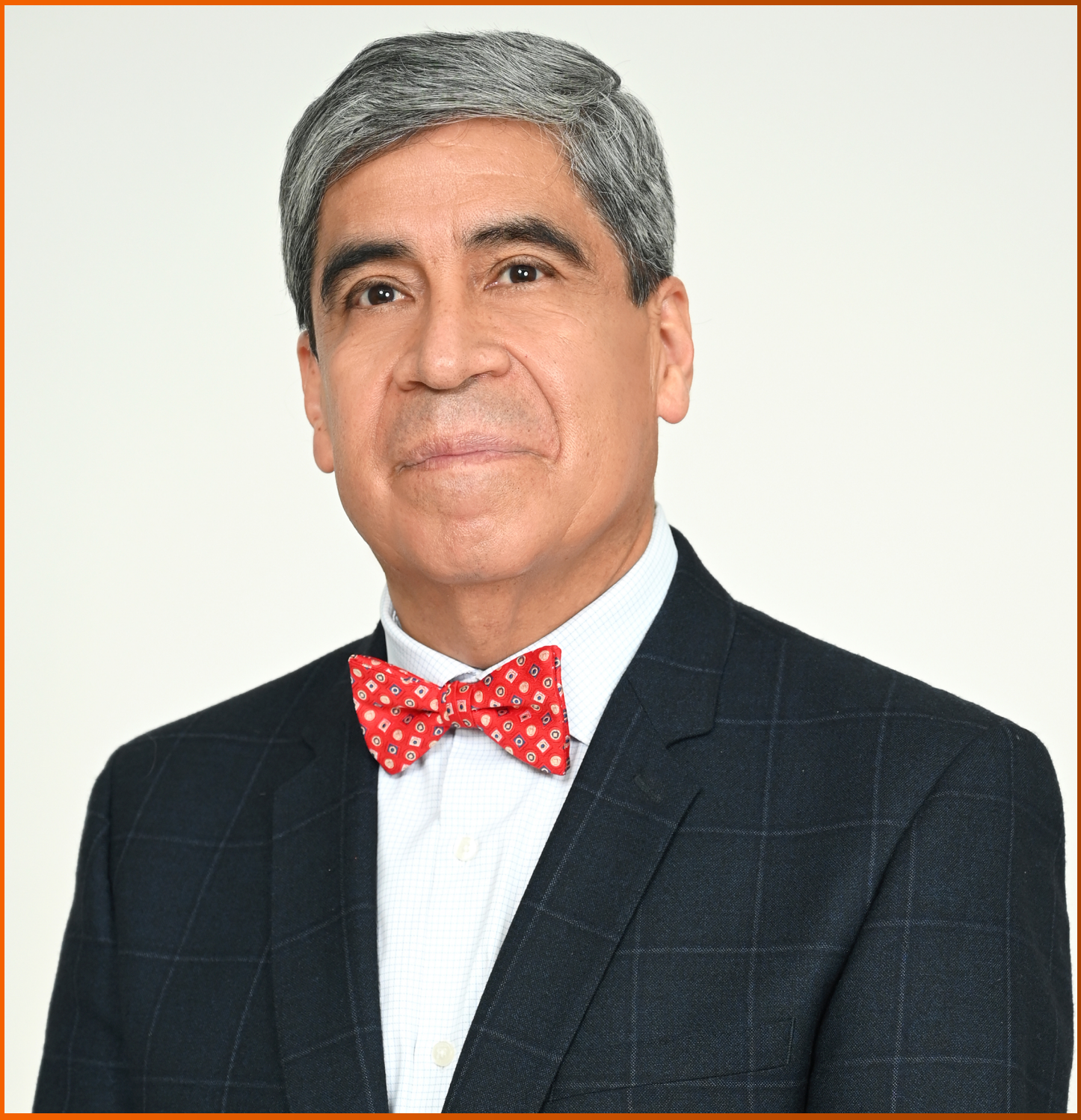

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ABOUT COVER

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REVIEW

Clinical relevance of intestinal barrier dysfunction in common gastrointestinal diseases

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Abstract

The intestinal barrier is a complex and well-controlled physiological construct designed to separate luminal contents from the bowel wall. In this review, we focus on the intestinal barrier's relationship with the host's immune system interaction and the external environment, specifically the microbiome. The bowel allows the host to obtain nutrients vital to survival while protecting itself from harmful pathogens, luminal antigens, or other pro-inflammatory factors. Control over barrier function and the luminal milieu is maintained at the biochemical, cellular, and immunological level. However, disruption to this highly regulated environment can cause disease. Recent advances to the field have progressed the mechanistic understanding of compromised intestinal barrier function in the context of gastrointestinal pathology. There are numerous examples where bowel barrier dysfunction and the resulting interaction between the microbiome and the immune system has disease-triggering consequences. The purpose of this review is to summarize the clinical relevance of intestinal barrier dysfunction in common gastrointestinal and related diseases. This may help highlight the importance of restoring barrier function as a therapeutic mechanism of action in gastrointestinal pathology.

Key Words: Intestinal barrier; Microbiome; Gastrointestinal disease; Inflammatory bowel disease; Inflammatory bowel syndrome; Colitis

Core Tip: Intestinal barrier dysfunction is an underlying pathophysiological feature in many gastrointestinal diseases. Understanding barrier dysfunction may help drive a deeper understanding of gastrointestinal pathology and identify novel way(s) to manage and/or treat disease. Here, we summarize the evidence supporting intestinal barrier dysfunction in common immune-mediated gastrointestinal diseases and its

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INTRODUCTION

The gut contains about 0.2 kg, or approximately 40 trillion cells, of bacteria (commonly referred to as the microbiome) and maintains a highly specialized architecture to manage the host's interaction with food-borne and bacterial antigens[1]. While the epithelial monolayer and multiprotein junctional complexes [*e.g.*, tight junctions (TJs)] comprise the primary physical barrier, other cell types, such as mucin-producing Goblet cells and Paneth cells, have antimicrobial functions[2,3]. The bowel's defense system is further supported by proximal lymphoid tissue, which is critical to maintaining homeostasis with respect to microorganisms beneficial to the host as well as responding to pathogenic invaders. This interconnected system facilitates a robust immune response and allows broad coverage over a large surface area. In fact, it is estimated that the intestinal lining contains more immune cells and produces more antibodies than any other organ[4].

Barrier maintenance is challenged by the rapid turnover of intestinal epithetical cells–occurring every 4-5 d[5]. Homeostasis is readily maintained, in part, due to cell shedding and stem cell proliferation within the intestinal crypts; however, loss of intestinal barrier function may expose antigens of the microbiome to immune cells inside luminal epithelium. This can activate an immune response and cause pathology. The purpose of this review is to characterize and quantify the clinical relevance of impaired intestinal barrier function in common gastrointestinal diseases. Specifically, we will review Crohn's disease (CD), ulcerative colitis (UC), diarrhea-predominant irritable bowel syndrome (IBS-D), and an emerging pharmacotherapy-associated condition known as immune checkpoint inhibitor-related colitis. In addition to reviewing the current state of knowledge, this review may help identify knowledge gaps in the understanding of impaired intestinal barrier function and highlight the relevance of restoring barrier function as a therapeutic mechanism of action.

CLINICAL ASSESSMENT OF BOWEL PERMEABILITY

Functional *in vivo* assessment of bowel permeability relies on the measurement of orally administered sugars, or some other indigestible probe, that reflects non-mediated absorption through the paracellular and/or transcellular route. Selection of an appropriate test probe involves knowledge of the molecules' properties, route(s) of permeation, and potential confounding variables-which have been described elsewhere[6]. A summary of clinically available probes is listed in Table 1.

Lactulose/mannitol

The lactulose/mannitol (L/M) test is the most widely used method to evaluate small intestinal permeability *in vivo*. The basis for a two-sugar test relies on the para- and transcellular absorption pathways of lactulose and mannitol. Mannitol is a monosaccharide approximately 6.5 Å in molecular diameter and is readily absorbed by passive diffusion through the membrane of bowel epithelium (transcellular transport). In contrast, the larger disaccharide lactulose (9.5 Å) is minimally absorbed because of its size; however in abnormal situations (*e.g.* pathology) lactulose can fit through intercellular spaces and becomes absorbed *via* the paracellular route (Figure 1)[7-9]. Following absorption into the blood stream, both sugars are then renally secreted by glomerular filtration without active re-absorption and are thus readily measurable in blood and urine. Because of the paracellular *vs* transcellular absorption pattern of lactulose and mannitol, the L/M ratio is small (or close to zero) under normal physiological conditions. In disease, alterations to lactulose and/or mannitol excretion

Table 1 Functional probes used to clinically assess bowel permeability in humans

Functional probe	Proposed test site	Sample site	Ref.
^{51}Cr-EDTA	Whole intestine	Urine	Bjarnason *et al*[14], 1983
Lactulose/mannitol[1]	Small intestine	Plasma/urine	Rao *et al*[11], 2011
PEG400	Whole intestine	Urine	Ma *et al*[132], 1990
Sucralose[2]	Colon	Urine	Anderson *et al*[133], 2004
Sucrose[2]	Gastroduodenal	Urine	Meddings *et al*[134], 1993

[1]Cellobiose and L-rhamnose have been used as alternatives to lactulose and mannitol, respectively.
[2]Usually in combination with lactulose/mannitol. ^{51}Cr-EDTA: Chromium-51-ethylenediaminetetra-acetate.

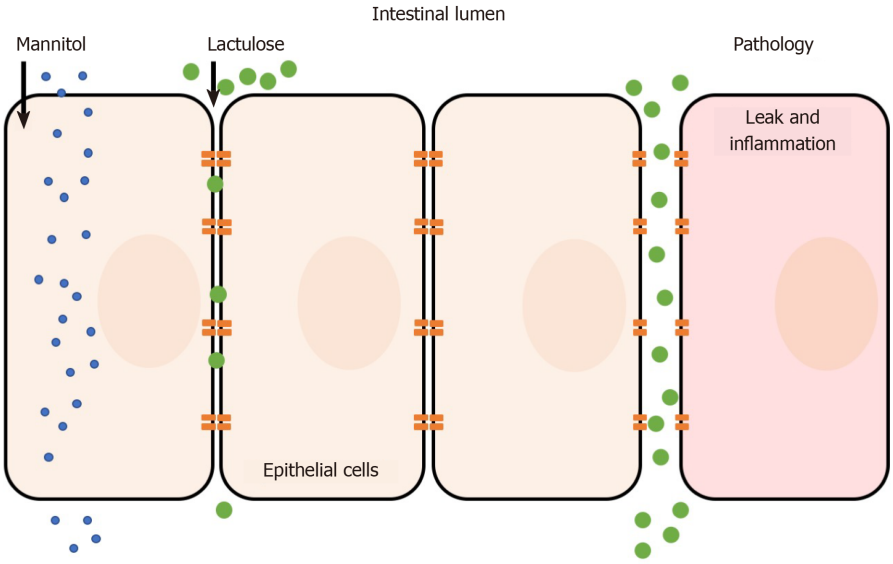

Figure 1 Absorption pathways of lactulose and mannitol.

are thought to reflect pathological features of leakiness (lactulose) normalized to surface area (mannitol)[10]. Hence, the L/M ratio is higher under pathological conditions due to increased paracellular absorption of lactulose. Both sugars cannot be metabolized by bacteria in the small bowel (but are by colonic bacteria) and are unaffected by differences in liver metabolism, which ensure its integrity is maintained for measurement[6]. The L/M test consists of concomitant oral administration of lactulose and mannitol (dissolved in water) followed by timed blood or urine collection (up to 24 h). Urinary excretion from 0 to approximately 5 h reflects small intestine permeability, although methodological enhancements of the L/M tests suggest that shorter time frames (*e.g.*, 0-2 h) may be ideal[11]. L/M can also be measured in serum to reduce complications of timed urine collections. For example, one dual sugar test was shown to have similar results when analyzing either serum or urine[12]. The serum-based test was simpler because it required less sample volume, needed a short waiting time after sugar ingestion (1 h compared to 5 h for urine collection), and was more cost-effective to analyze than urine[12]. The L/M test has also shown intra-individual and analytical repeatability, making it a simple and reliable measurement method[13].

The primary results of the test are expressed as lactulose-to-mannitol ratio (*e.g.* lymphocyte-to-monocyte ratio (LMR) in urine: %lactulose/%mannitol where "%" represents the percentage of the ingested dose) with a higher LMR indicating increased small intestine permeability. Other sugars have been investigated (*e.g.* L-arabinose, L-rhamnose, raffinose) but there appears to be no widely-accepted practical benefit over L/M[6].

Chromium-51-ethylenediaminetetra-acetate

Chromium-51-ethylenediaminetetra-acetate (^{51}Cr-EDTA) is a radiolabeled form of the

indigestible EDTA. ^{51}Cr-EDTA is orally administered and transverses the lumen paracellularly, similar to large sugars, where it is readily excreted in urine[14]. ^{51}Cr-EDTA is believed to cross both the small and large intestine, in contrast to lactulose and mannitol, which predominately transverses the small intestine only[15]. Moreover, when combined with a sugar probe to correct for small intestinal absorption, ^{51}Cr-EDTA allows assessment of colonic permeability because it is not metabolized by the microbiome of the colon. Routine usefulness of the test, however, appears to be limited by reliability and practicality (*e.g.*, up to 24 h urine collection, requires gamma counter, short shelf-life of ^{51}Cr)[6,14].

Confocal laser endomicroscopy

Confocal laser endomicroscopy is an *in vivo* real-time imaging method that can detect and quantify mucosal abnormalities at the microscopic level following administration of a contrast agent. It has several clinical applications such as in the identification of dysplasias/neoplasias, diagnostic work-up of Inflammatory bowel disease (IBD), and other surgical use cases. Confocal laser endomicroscopy has been also experimentally applied to assess intestinal permeability[16,17]. The main advantage is that it can quantify permeability at specific intestinal site(s), which may be cross-referenced with histological or endoscopic findings. However, the technique remains experimental and there appears to be no well-validated methodology or robust reproducible findings in the literature[18]. Technical and analytical challenges may have also contributed to its lack of wide spread acceptance.

IBD

IBD is a group of chronic gastrointestinal disorders primarily consisting of CD and UC. Genetic susceptibility and environmental triggers underly the etiology of pathology which converge to activate an immune response associated with compromised intestinal barrier[19-21]. For example, nucleotide-binding oligomerization domain 2 (NOD2), expressed in ileal Paneth cells, is induced in response to the presence of bacterial components and activates an innate immune response; genetic analysis has confirmed the relationship between mutations in NOD2 and CD and it is estimated that 30%-50% of CD patients in the Western hemisphere harbor one mutant allele of NOD2[22-24]. Two pathogenic alleles increases the risk of CD by 20-40 times[23,24]. These data suggest that certain susceptibility factors may contribute to dysfunctional/overactive innate and adaptive immune pathways that may not be able to self-regulate as it would under normal or homeostatic conditions. Substantial data implicate abnormal bowel barrier function as an underlying pathological feature of IBD.

CD

CD is a chronic relapsing-remitting inflammatory disease of the gastrointestinal tract, the cause of which remains generally unknown. The disease affects the gastrointestinal tract discontinuously from mouth to anus, but most commonly the disease is located both in ileum and colon (40%), followed by a disease in the small bowel only (30%), and in the colon only (25%). CD can lead to severe disability and significant reduction in quality of life[25,26]. Abnormal intestinal barrier function has been a well-established pathological feature of CD for nearly 40 years[27-31]. Impaired intestinal function may be multifactorial but structural barrier damage appears to be an characteristic pathological feature (Table 2).

The intestinal barrier, as it relates to bowel epithelial function, consists of a polarized monolayer of epithelial columnar cells connected by TJs, an intercellular feature designed to create separation between gut and lumen. Four groups of proteins comprise TJs: Occludin tricellulin, junctional adhesion molecule, and claudins[32-35]; The latter appear to have a particular crucial role in barrier function including seal or pore-forming properties and may act as paracellular channels for small ions[36-39]. The relevance of TJ abnormalities in CD has been demonstrated in sigmoid colon biopsies, which revealed a reduction in the number of TJ strands, reduced depth of TJ meshwork, and strand breaks[40]. Expression of sealing claudin-3, -5 and -8 and occludin were diminished, indicating altered TJ structure[40-42]. The pore-forming claudin 2 also appears to be upregulated[40,43]. These data were generated from patients with mild-to-moderate CD, suggesting that impaired TJ structure may characteristic that occurs early in disease progression. This is consistent with the hypothesis that permeability abnormalities are a primary disorder, as evidenced by healthy first-

Table 2 Clinical intestinal permeability function in gastrointestinal diseases

Disease	Disease activity	Functional probe	Intestinal permeability change	Ref.
CD	Not reported	C/M	↑	Secondulfo *et al*[135], 2001
	Not reported	^{51}Cr-EDTA	↑	Jenkins *et al*[136], 1987
	Low activity and high activity	Iohexol	Low activity: ↑; high activity: ↑	Gerova *et al*[55], 2011
	Low activity and high activity	L/M	Low activity: ↑; high activity: ↑	Benjamin *et al*[57], 2008
	Remission, low activity, and high activity	L/M	Remission: ↔; low activity: ↑; high activity: ↑	Welcker *et al*[82], 2004
	Remission	L/M	↑	Wyatt *et al*[52], 1993
	Low activity	L/M; L/R; R/M	L/M: ↑; L/R: ↑; R/M: ↔	Katz *et al*[30], 1989
	Low activity	L/M; L/R; L/PEG; PEG/M; PEG/R	↔	Munkholm *et al*[45], 1994
UC	Not reported	^{51}Cr-EDTA	↑	Jenkins *et al*[136], 1987
	Remission, low activity, and high activity	C/M; C/R; L/M; L/R;	Remission: ↔ or ↑[1]; low activity: ↔ or ↑[1]; high activity: ↑	Welcker *et al*[82], 2004
	Low activity and high activity	Iohexol	Low activity: ↑; high activity: ↑	Gerova *et al*[55], 2011
	Remission	L/M; S; Su	↑	Büning *et al*[83], 2012
	Remission	L/M/S/Su/E/R	↔[2]	Wegh *et al*[81], 2019
IBS-D	Active	^{51}Cr-EDTA	↑	Gecse *et al*[103], 2012
		^{51}Cr-EDTA	↑	Dunlop *et al*[102], 2006
		L/R	↑	Mujagic *et al*[105], 2014
		L/M	↑	Shulman *et al*[98], 2014
		L/M	↑	Vazquez-Roque *et al*[100], 2012
		L/M	↑	Zhou *et al*[104], 2009
		L/R	↑	Zhou *et al*[137], 2010

[1]Increased or no change depending on the functional test.
[2]Study did not include a true (healthy) control arm. ^{51}Cr-EDTA: Chromium-51-ethylenediaminetetra-acetate; C: Cellobiose; CD: Crohn's disease; E: Erythritol; IBS-D: Diarrhea-predominant irritable bowel syndrome; L: Lactulose; M: Mannitol; PEG: PEG400; R: L-rhamnose; S: Sucrose; Su: Sucralose; UC: Ulcerative colitis; ↑: Increase; ↔: No change.

degree relatives of CD patients who display detectable permeability defects and who also have a higher risk of developing CD compared to healthy non-relatives[44-47]. Zeissig *et al*[40] (2007) also discovered that epithelial apoptosis was increased in mild-to-moderate CD, a feature previously thought to be specific to UC[40]. These data implicate that both changes to TJ structure and epithelial apoptosis may serve as a mechanistic explanation(s) consistent with barrier dysfunction. This hypothesis is also consistent with less pronounced changes to TJ structure and epithelial apoptosis in patients in remission, suggesting that epithelial structure may correlate with clinical symptoms, although permeability alterations have been observed in areas evident of lesions in addition to areas absent of macroscopic injury[48,49].

Increased intestinal permeability in patients with CD is well-recognized and has also been shown to associate with symptomatic status in patients with IBD. In one prospective study, patients with symptomatic IBD had a significantly higher median Confocal Leak Score (CLS) (19.0) than patients with asymptomatic IBD (7.3; $P < 0.001$) or control subjects (5.9, $P < 0.001$)[50]; no differences in CLS were found between control subjects and asymptomatic IBD patients, suggesting that low barrier function may be a causative mechanism underlying IBD symptoms. This was confirmed on a symptom level when regression analysis revealed that every increase in CLS of 1.9 correlated with an additional diarrheal motion per day[50]. Other studies have found a relationship between increased intestinal permeability in clinically inactive patients and also demonstrated a correlation with pending disease relapse[51,52]. Wyatt *et al*[52] (1993) prospectively followed 72 CD patients in symptomatic remission and following

a L/M test, discovered the permeability index (PI) was significantly higher in patients than in controls at baseline (0.046 *vs* 0.018, respectively)[52]. After one year, the relapse rate was 70% in CD patients who had an abnormal PI compared to 17% of patients who had a normal PI. The sensitivity of the PI as predictor of relapse was 81% and specificity was 73%. Other studies are consistent with these findings and have estimated relative risk of relapse within 1 or 2 years in patients with elevated intestinal permeability to be approximately 3-18[7,17,51,53]. This may be explained, in part, by the association between increased intestinal permeability, inflammation, and disease activity (*i.e.* Crohn's disease activity index)[54,55]. In addition, increased epithelial gaps as measured by confocal laser endomicroscopy appear to predict hospitalization or surgery in patients with IBD[56]. These data point to elevated bowel permeability that may allow for a greater probability of interaction between certain pathogens of the microbiome with the epithelial immune system of the bowel.

Elevated intestinal permeability may also indicate greater disease involvement. For example, more patients with ileo-colonic disease (57.8%) had an abnormal PI as measured by L/M compared to 26.7% and 15.6% of patients with colonic and small intestinal disease, respectively[57]. The authors also found that patients with stricturing disease had a significantly elevated LMR compared to patients with non-fistulizing non-stricturing disease. These data highlight the clinical relevance of intestinal permeability measures in the context of disease involvement, symptoms of the IBD disease, and as trigger for relapse that may suggest restoring barrier function may be a useful approach to maintain remission[7,52,53,58,59]. It should be noted that not all studies have found intestinal permeability abnormalities in CD[45]. Although the reasons for this observation are not clear, differences in study design, study population, selected probe, sample size, and analytical methods, among others, may contribute to conflicting literature reports.

As noted earlier, increased intestinal permeability may or may not occur in the presence of histological findings. Protein antigen uptake in macroscopically normal segments of distal ileum of patients with CD was reported to be increased despite being histologically unaffected[60]. Early histological lesions appear to occur in the follicle-associated epithelium (FAE) of lymphoid follicles and Payer's Patches located in the mucosa, which can occur even in the presence of normal overlying epithelium[61,62]. Indeed, Keita *et al*[63] (2008) discovered enhanced intercellular and transcellular uptake of E. Coli across FAE in ileal CD, but not UC, indicating defective barrier function at immunological inductive sites[63]. This appears to be related to microtubule-, microfilament-dependent internalization and transcytosis of bacteria associated with enterocyte cytoskeletal changes under conditions of stress–further linking structural changes to increased permeability[64]. *In vivo* studies support bacterial translocation secondary to increased paracellular permeability as the mechanistic basis for chronicity and/or acute relapses in IBD[65]. These examples may help explain the relevance of barrier function and associated functional tests in the absence of endoscopic and/or histological findings.

Most available treatments in CD aim to control symptoms (*e.g.*, thiopurines, and steroids) or reduce the immune response, for example by inhibiting tumor necrosis factor-α (TNF-α) (*e.g.* infliximab, adalimumab, golimumab, and certolizumab pegol)[66]. Although anti-TNF-α agents have proven clinically beneficial, approximately 1 in 3 do not respond to induction therapy, and of those who do respond, approximately 1 in 3 become secondary non-responders during maintenance therapy[67-71]. Anti-TNF-α agents are also associated with several side effects (*e.g.* risk of infection, malignancies, skin lesions, immune reactions, peri-operative complications, and decreased fertility)[72-75]. Persistent diarrhea in patients with CD despite mucosal healing (Mayo scores of 0-1 or segmental endoscopic severity CD scores of 0-5) is not uncommon suggesting that achieving mucosal healing alone through conventional management may be insufficient in some patients. This may warrant developing therapies targeted to intestinal barrier dysfunction and dysbiosis[76]. We suggest that next generation treatments for CD should focus on restoring bowel barrier function as a way to induce and maintain remission which may also prevent some of the side effects of long-term general immunosuppressive therapy.

UC

Similar to CD, the pathogenesis of UC appears to be related to both genetic and environmental susceptibility factors. UC differs in that it continuously affects the colon and perirectal region with shallow and indiscrete ulcers associated with inflammation limited to the mucosa. Structural characterization of the epithelia in UC appears less robust compared to the available evidence available in CD; however, some notable defects have been reported. Freeze-fracture electron microscopy revealed epithelial cell

TJ strand count was reduced by approximately 30% in UC biopsies compared to controls[77]. This paralleled the authors findings that found a 50% and 80% reduction in total and epithelial wall resistance, respectively, using alternating current impedance analysis, which indicates a leak-flux imbalance that may help explain diarrhea in UC[77]. Similar studies corroborate these reports and also suggest that apoptotic foci are a source of epithelial leaks that appear more pronounced with increasing inflammation[78]. Indeed, Th2 cytokines, *e.g.*, interleukin (IL)-13, have been shown to be important effector molecules relevant to epithelial dysfunction that affect tight junctions and cellular apoptosis[79]. Cytokine-mediated altered expression of TJs appears to occur at the signal transduction level[80].

In contrast to CD, patients with UC and in remission generally do not always appear to have notable permeability defects (Table 2). Wegh *et al*[81] (2019) reported that when using a 5-sugar test, intestinal permeability in patients with UC in clinical remission was not different compared to historic values (*e.g.*, lactulose/rhamnose approximately 0.02-0.06), although the study did not include a true control arm[81]. Urinary sucrose excretion and sucralose/erythritol ratio also appeared normal. This was consistent with normal or slightly abnormal inflammatory markers in the study population, suggesting that functional permeability tests may have little relevance in subclinical disease particularly in the absence of abnormal biomarkers. Similar findings were observed by Welcker *et al*[82] (2004) when using multiple functional probes[82]. In a larger study including patients with UC in remission and healthy first degree relatives however, an elevated LMR was observed significantly more often in UC patients in remission (28.1%) compared with healthy controls (6.1%; $P < 0.001$)[83]. This is somewhat paradoxical as UC affects the colon, and gastroduodenal and colonic permeability were found to be normal using sucrose and sucralose as functional markers, respectively. However, healthy first degree relatives also had an higher prevalence of elevated LMR compared to controls (20% *vs* 6.1%, respectively, $P = 0.01$), possibly indicating that abnormal permeability may be a risk factor rather than a reliable marker of disease, at least in patients in remission[83].

Despite histological and *in vitro* evidence indicating colonic barrier abnormalities, clinical data that reliably demonstrate altered barrier function in active UC appears less clear compared to CD. This may be related to the observation that intestinal permeability is in UC less prevalent than in CD[55]. Welcker *et al*[82] (2004) for example reported increased permeability in patients with low and high activity UC[82]. However, the interpretation of this evidence is potentially limited by the use of multiple functional tests (multiplicity) and low sample size (*e.g.*, $n = 4$ in patients with highly active UC). Further research is needed to clarify the magnitude, clinical relevance, and true anatomical location(s) of impaired barrier function, particularly in active UC.

The therapeutic goals of UC are similar to CD: Induce and maintain remission. Biologic agents including anti-TNF-α agents, Janus kinase inhibitors, IL-12/IL-23 inhibitors, and integrin receptor antagonists are considered when conventional therapies (*e.g.* aminosalicylates, oral immunomodulators, and corticosteroids) are inadequate. Because these agents are immune-targeting, they carry traditional down side risks of potentially long-term immunosuppression and can have notable contraindications (*e.g.* in patients with advanced heart failure or neurological conditions)[84].

IBS

IBS is primarily characterized by recurring abdominal pain and bowel movement changes without discernable gross pathology[85]. Patients experience mixed symptoms such as pain, bloating, abnormal stools, and dyspepsia and are categorized by stool pattern: Diarrhea predominant (IBS-D), constipation predominant (IBS-C), or mixed (IBS-M). IBS is highly prevalent (global prevalence: Approximately 10%) and negatively affects quality of life, work productivity, and can even cause severe disability[86-88].

The pathophysiology of IBS appears multifactorial. Historically, environmental and psychosocial triggers, genetic modifiers, intestinal dysmotility, microbiota disturbances, and bile acid malabsorption, among others, had been proposed[89-93]. However, more recently IBS is thought to be a disease of the gut-brain axis with the trigger being an interaction between the microbiome and certain immune cells[94]. Mast cells, for example, have been shown to correlate with intestinal permeability in patients with IBS-D and are a key mediator of downstream inflammation that are also

linked to symptoms such as pain[95,96]. Emerging evidence continues to support mast cells as central to the initiation and symptomatic presentation of IBS.

Gastroenteritis (especially related to *Campylobacter jejuni* infection) has been shown to precede or cause IBS symptoms, particularly in IBS-D, suggesting that alterations to the intestinal milieu and subsequent immune stimulation may be a primary driver contributing to IBS-D pathology[91,97]. Spiller *et al*[97] (2000) was among the first to report increased gut permeability (approximately 4-fold increase in LMR compared to controls) in a small cohort of patients with post-dysenteric IBS[97]; immunohistology also revealed elevated CD3, CD4, and CD8 lymphocyte counts in the lamina propria, consistent with the known relationship between the immune system and gut permeability. Subsequent studies have confirmed these observations[91,98-100]. Thus, impaired intestinal barrier function and subsequent microbial infiltration have become central to the proposed gut disease model in certain forms of IBS[101]. The pathogenesis of IBS also appears to link a relationship between the microbiome, gut, and brain, as neural networks have been shown to be influenced by the composition of the gut microbiome[94]; therefore the gut-brain axis is highly relevant in the pathophysiology of IBS.

IBS-D

Increased intestinal permeability appears greatest in IBS-D. Dunlop *et al*[102] (2006) showed that proximal small intestinal permeability, as measured by ^{51}Cr-EDTA, was significantly higher in patients with postinfectious IBS-D compared to patients with IBS-C and healthy control subjects[102]. Patients with IBS-D but without a history of acute gastroenteritis had the highest median excretion of ^{51}Cr-EDTA in the study, further implicating the diarrhea-predominant phenotype with impaired intestinal barrier function; colonic permeability has been shown to correlate with stool frequency, suggesting site-specific defects in barrier function may be related to disease severity[103]. Similar observations have been reported in the literature[104,105]. IBS-C was not included in this review because intestinal permeability appears to be normal in this patient population[106].

The relationship between intestinal permeability and symptoms was evaluated by Gecse *et al*[103] (2012) who used ^{51}Cr-EDTA as an intestinal permeability probe in patients with IBS-D[103]. The number of stools per week was significantly correlated with permeability (Pearson $r = 0.62$; $P = 0.0057$). Interestingly, ^{51}Cr-EDTA excretion was only elevated compared to control when measured between 5 and 24 h after ingestion, suggesting that colonic, but not small intestine, permeability may explain the study findings and indicates that site-specific permeability may be a feature of IBS-D[103]. ^{51}Cr-EDTA excretion was similar to patients with UC, suggesting that impaired bowel function in IBS-D may be more similar to IBD than IBS-C. It should also be noted that molecular markers of impaired intestinal barrier function help further establish the mechanistic association with IBS-D. These data indicate a strong link between intestinal barrier dysfunction, IBS-D, and disease severity. This shows that there is a good correlation between IBS-typical symptoms and the degree of increased bowel permeability. These data also indicate that the pathophysiology of IBS-D seems to differ from IBS-C.

Other measures of symptomatic disease severity, including visceral and thermal hypersensitivity to pain [visual analog score (VAS) and bowel severity (FBDSI score)], have been shown to correlate with intestinal permeability. Zhou *et al*[104] (2009) reported that IBS-D patients with a LMR of ≥ 0.07 had significantly higher VAS intensity ratings to nociceptive and thermal pain than those IBS patients and controls with a LMR of < 0.07[104]. The authors used nociceptive and thermal stimuli in areas apart from the gut because many patients with IBS frequently complain of pain in body regions somatotopically distinct from the gut. The authors hypothesized their findings may be related to sensitization of the myenteric plexus and the common spinal segments of the central nervous system, thus establishing a quantitative link between bowel permeability and pain symptoms. If true, restoring bowel permeability may also have an effect on pain.

In total, these data suggest that a therapy designed to restore intestinal barrier dysfunction may be a promising therapeutic approach in IBS-D. Similar to other gastrointestinal diseases discussed in this review, notable architectural defects in the intestinal architecture have been reported in IBS-D including decreased expression of transmembrane (*e.g.*, occludin and claudin-1) and intercellular TJs (*e.g.* ZO-1)[107-109]. It should be noted that mood disorders are also correlated with IBS; epidemiological evidence indicates that mood disorders develop after IBS in approximately 50% of patients, implicating gut mechanisms as drivers of non-gastrointestinal symptoms. Interestingly, one study found that the LMR correlated with IBS, interference with

activities and work and retrospectively measured anxiety and depression[98]. If true, correcting intestinal pathology (*e.g.*, intestinal barrier dysfunction) could also have a significant impact on mood disorders secondary to IBS[93,110,111].

Alosetron (5-HT3 receptor antagonist), rifaximin (antibacterial), and eluxadoline (mu-opioid receptor agonist) are among the few drugs with marketing authorization for IBS-D in the United States market. Alosetron was removed from the market from 2000-2002 due to safety reasons but subsequently re-introduced with a more restrictive label. The efficacy of approved agents also appears limited. For example, a meta-analysis of five rifaximin trials indicated a small improvement in global IBS symptoms when patients were treated with rifaximin (42.2%) compared to placebo (32.4%)[112]. Other agents commonly used include antidiarrheals (*e.g.*, loperamide), bile acid sequestrants (*e.g.* cholestyramine), antispasmodics (*e.g.*, dicyclomine), and antidepressants (tricyclic agents, *e.g.*, amitriptyline)[113]. Nonpharmacological interventions such as probiotics and diet modification are also considered[114,115]. The lack of robust, and well-controlled randomized trials makes it difficult to precisely quantify the efficacy and safety of all available options; however, consensus opinion and available data indicate that most patients do not achieve complete symptom relief[113,116]. Given the newly established pathophysiologic relationship between the microbiome and interactions with certain immune cells, it seems promising to develop interventions that repair and maintain bowel permeability as next generation treatments for IBS-D.

IMMUNE CHECKPOINT INHIBITOR COLITIS

Immune checkpoint inhibitors (ICIs) are a class of monoclonal antibodies designed to interrupt critical signaling pathways between T cells and antigen-presenting cells. At least 6 approved ICIs have proven clinically effective across more than several dozen oncology indications. Despite their robust efficacy, ICIs are associated with a wide range of toxicities. The most common type of adverse events is a group of immune-mediated conditions including colitis, hepatitis, and thyroiditis, and generally appear early following treatment initiation. As these conditions resemble autoimmune diseases, this seems to suggest an abnormal or exaggerated immune response, possibly in relation to antigen exposure, such as the microbiome. Among immune-mediated diseases, gastrointestinal toxicity (ICI colitis) is the most common adverse event associated with ICI therapy that occurs usually in 6-8 wk after initiation of treatment[117]. Diarrhea and colitis occurs in up to approximately 54% and 22% of patients treated with anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) therapy, respectively[118]. programmed cell death protein 1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) inhibitors appear to have lower rates of diarrhea and colitis (up to 30% and 7% respectively), although the incidence of these toxicities is high in anti-CTLA4 and anti PD-1/PD-L1 combination therapy (45%), with approximately 10% of cases reported as grade 3 or higher[117,118].

The endoscopic and histologic features of ICI-related colitis appear to resemble IBD[117,119,120]. For example, in 39 patients with ipilimumab-induced colitis, immune infiltrate was highly prevalent: Neutrophilic infiltrate occurred in 46% of patients, lymphocytic infiltrate in 15% of patients and a mixed neutrophilic–lymphocytic infiltrate 38% of patients[120]. Edema, erythema, erosions and ulceration are also common endoscopic features. The same study by Marthey *et al*[120] (2016) found that 97% patients with ipilimumab-induced colitis had erythema or ulceration in the sigmoid colon or rectum and 66% had extensive colitis[120]. The pathogenesis of ICI-related colitis, therefore, appears to relate to colonic inflammation although the precise mechanism(s) are not well characterized. Given clinicopathological similarities IBD, it is reasonable to hypothesize that impaired bowel barrier function may play a role in the development of colitis. This is supported by a retrospective study that found pre-existing IBD increases the risk of gastrointestinal adverse events following ICI therapy[121]. Samaan *et al*[117] (2018) proposed several barrier function-related etiopathogenic hypotheses including pre-existing intestinal dysbiosis or epithelial stress/mucositis secondary to chemotherapy[117]. Thus, normalizing bowel barrier function may be a promising therapeutic mechanism to treat or even prevent ICI-related colitis.

NON-GASTROINTESTINAL DISEASES

While the relationship between impaired bowel barrier function and bowel pathology appears self-evident, increasing evidence has also revealed a connection between the bowel and the brain–colloquially referred to as the gut-brain axis. This axis consists of complex, bidirectional pathways relevant to normal gastrointestinal functions (*e.g.* satiation), but also to higher executive functions (*e.g.* decision making) and emotions (*e.g.* fear and stress)[122]. Communication between the bowel and the brain is regulated at the neuronal, endocrine, and immunological level. Hence, as the lumen interfaces with the microbial environment, including signaling molecules such as quorum-sensing molecules and bile acids within the lumen, communication to the brain can reflect interaction(s) between the host and the microbiome[123]. Substantial nonclinical and clinical evidence has revealed a well-documented connection between impaired bowel barrier function, microbial dysbiosis, and neurological diseases[123,124]. For example, a recent nationwide longitudinal study revealed the hazard ratio of developing dementia among patients with IBD was 2.54[125]. In Parkinson's disease, there is increasing evidence that pathogenic microbial peptides are able to access the enteric nervous system and/or systemic circulation *via* a highly permeable bowel wall, which could act as a disease trigger or driver of neuronal destruction, possibly by way of retrograde axonal and transneuronal transport of α-synuclein *via* the vagus nerve[126]. Increased bacteria/endotoxin exposure has been shown to correlate with sigmoid mucosa α-synuclein in Parkinson's disease, consistent with findings of increased intestinal permeability in patients with Parkinson's disease[127]. Altered intestinal permeability has been linked to other extraintestinal disease such as nonalcoholic fatty liver disease, type 1 diabetes, arthritis, and other autoimmune diseases[128-131]. This may imply that restoring intestinal barrier function may have therapeutic benefits beyond bowel pathology alone.

CONCLUSION

Multiple genetic, environmental, and host-related risk factors play a pivotal role in the development of gastrointestinal pathology, yet the initiation of pathology coupled with the complex interplay between the microbiome and host makes the precise pathophysiology of disease difficult to assess at the individual level. Impaired intestinal barrier function appears to be a central characteristic of common gastrointestinal diseases as summarized in this review. The field of gastroenterology continues to evolve as a more precise characterization of intestinal barrier function in the context of gastrointestinal disease is uncovered. This will improve our ability to understand the complex role of intestinal barrier abnormalities and design therapeutic interventions to restore barrier function. Novel therapies may be developed to target bowel permeability which address a primary disease trigger without the side effects of traditional long-term immunosuppressive therapy.

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