VISCERAL ANTINOCICEPTIVE AND PROINFLAMMATORY EFFECTS OF PROTEINASE-ACTIVATED RECEPTOR-4 AND ITS ROLE IN THE PATHOGENESIS OF ULCERATIVE COLITIS

Ph.D. Thesis

Anita Annaházi M.D.

First Department of Medicine,

University of Szeged,

Szeged, Hungary

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IV. **Annaházi A**, Gecse K, Dabek M, Ait-Belgnaoui A, Rosztóczy A, Róka R, Molnár T, Theodorou V, Wittmann T, Bueno L, Eutamene H. Fecal Supernatants from Ulcerative Colitis Patients Display Antinociceptive Effects On Colorectal Sensitivity When Infused Intracolonically in Mice: Role of Cathepsin-G. *Gastroenterology* 2009; 136, (Suppl. 1), A19

V. Dabek M, Ferrier L, **Annaházi A**, Róka R, Gecse K, Wittmann T, Fioramonti J, Theodorou V, Bueno L. Intracolonic Infusion of Fecal Supernatant from UC Patients Triggers Altered Permeability and Inflammation in Mice: Role of Cathepsin G and Protease-Activated Receptor-4. *Gastroenterology* 2009; 13*6, (Suppl. 1), A*242

VI. **Annaházi A,** Dabek M., Gecse K, Rosztóczy A, Róka R, Theodorou V, Wittmann T, Eutamene H, Bueno L. Endogenous Antinociceptive Role of Par-4 in Inflammatory but Not in Stress-Induced Colorectal Hyperalgesia in Mice. *Gastroenterology* 2010, 138; 5, (Suppl. 1), S-574.

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1. INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD), collectively known as inflammatory bowel diseases (IBD) are chronic inflammatory gastrointestinal disorders affecting estimatedly 2.2 million persons in Europe (57). IBD is responsible for a significant patient morbidity, decreased quality of life and increased occurrence of colorectal cancers. Despite their high clinical significance, the pathogenesis of these diseases is only partially understood. An early study have found 10-fold increase in the occurrence of IBD among first degree relatives of such patients, underlining the role of genetic factors (72). Since, it has been clearly established that a dysregulated mucosal immune response to antigens develops in the genetically susceptible host. However, environmental factors are also important, as penetrance in monozygotic twins is only 50% for CD and 20% for UC (40).

Increased intestinal permeability has been demonstrated more than twenty years ago in an experimental colitis model (83) and in IBD patients (48). Since that, a growing body of evidence demonstrates that besides genetic predisposition, luminal factors participate to trigger colonic inflammation in IBD and particularly in UC (60, 92). It has been shown by animal models that intestinal inflammation can be initiated by molecular defects in the colonic epithelium even in the presence of intact bacterial flora or immune system (69, 88), as increased permeability may facilitate the passage of luminal antigens into the mucosa, therefore may trigger an exaggerated immune response, an increased cytokine-production and a neutrophil infiltration (80). In case of experimental colitis, mucosal barrier dysfunction facilitates the permeation of bacterial antigens, causing the elevation of systemic endotoxin levels, therefore aggravating colitis (34). Human studies demonstrated that increased intestinal permeability can predict relapse in inactive CD patients (4, 93), and it was detected also in their first degree relatives, suggesting that it can be a pathogenetic factor, not only the consequence of gut inflammation (28, 59). Examinations on UC patients evidenced that disease severity correlates with the increase in intestinal permeability, and effective treatment restores permeability changes in these patients (5). Partly, epithelial barrier dysfunction may be explained by tight junction alterations, which are present in the colonic epithelium in active CD and UC (42, 43, 96).

Tight junctions are highly dynamic structures composed by a multiprotein complex (70), that undergo rapid regulatory changes during inflammation (42). Tight junction opening is driven through myosin light chain (MLC) phosphorylation, which depends on myosin light chain kinase (MLCK) activation (66). Further, decreased expression of tight junction proteins is observed in IBD patients, particularly with changes in expression and distribution of occludin (54) and claudins 2, 5 and 8 (96).

The decreased expression of occludin is associated with the transmigration of neutrophils in the colon of UC patients (54).

In a recent study, a new factor has been identified in the feces of UC patients that may play a role in the pathogenesis of the disease, namely cathepsin G (Cat-G), a serine-protease of the neutrophil granulocytes which belongs to the peptidase S1 protein family (29). The acute phase of UC is characterized by mucosal inflammation with excessive migration and degranulation of neutrophils in the injured area. (76). Neutrophil accumulation within epithelial crypts and in the intestinal lumen directly correlates with clinical disease activity and epithelial injury (23, 41). Neutrophils migrate through the colonic wall, and are detectable in the stool of UC patients (82). Moreover, granulocyte and monocyte/macrophage adsorptive apheresis reduced clinical activity index in UC patients (41). Neutrophils can release a large number of proteins from their major granule populations, primary (azurophilic) and secondary (specific) granules. Secondary granules contain lactoferrin and cathelicidin, primary granules are filled with proteolytic enzymes and different bactericidal proteins such as Cat-G, elastase, myeloperoxidase (MPO), and lysozyme (17). These enzymes act together with reactive oxygen species in the degradation of engulfed microorganisms, furthermore, they exert various functions when excreted from cells during inflammatory processes These secreted products appear also in the stool, as polymorphonuclear elastase and calprotectin levels were significantly increased in fecal supernatant of UC patients compared to either healthy or IBS-D (35). Among these enzymes, Cat-G is of high importance, as it represents approximately 20% of the azurophylic proteins, and it plays roles in neutrophil function during inflammatory processes, such as degradation of extracellular matrix components and cytokines, modulation of integrin clustering on neutrophils, and direct chemoattraction of T cells and other leukocytes (84). Additionally, anti-cathepsin G antibodies were detected in 40.6% of 32 patients with active UC, and their prevalence was significantly higher in patients with severe colitis than in those with mild or moderate colitis (55). In a recent study from Dabek et al. (29), it has been demonstrated by RT-PCR and Western blot that Cat-G expression is higher in biopsies from UC patients than in that of controls. Further, elevated Cat-G activity has been found in fecal supernatant of UC patients compared to healthy subjects and IBS-D patients by an enzymatic assay.

Protease signaling is highly important in the gastrointestinal tract which is a rich source of proteases that originate from digestive secretions, inflammatory cells and resident microorganisms (3). Besides its nonspecific properties, Cat-G can activate a particular receptor, proteinase-activated receptor-4 (PAR-4), which was referred to as the “Cat-G receptor” (80) on platelets. PARs represent a family of 7-transmembrane G-protein-coupled receptors which is activated by the cleavage of its N-terminal domain by serine-proteases. The cleavage of the activation site reveals a new amino

terminal sequence consisting of a tethered ligand which can activate the receptor itself (44). To date, four PARs have been described, PAR-1,-2,-3 and -4. Conventionally, PARs can be activated by several proteases secreted by inflammatory cells, digestive glands and microorganisms during physiological and pathological conditions (3). PAR-1, 3 and 4 can be cleaved by thrombin, while PAR-2 and 4 are trypsin receptors. PAR-1,-2 and -4 can be activated also by synthetic peptides, called PAR-activating peptides (PAR-APs), with sequences based on their tethered ligand. PARs play an important role in gastrointestinal physiology and pathophysiology, but their role in the maintenance of mucosal integrity and in the pathogenesis of mucosal inflammation is not yet clarified (51). Activation of PAR-2 elicits permeability increase and inflammation in the colon of mice (19, 35), and the expression of PAR-2 was found elevated by immunohistochemistry in colonic tissue from UC patients compared to controls (53). PAR-4 is present in the rat colon (45), but also in human small and large intestine (94). Similarly to PAR-2, Dabek et al. have shown elevated PAR-4 expression in colonic biopsies from UC patients compared to those from control subjects. Further, in UC biopsies the internalization of PAR-4 was observed, suggesting the activation of this receptor on epithelial cells. These findings pointed out that Cat-G may be a factor involved in UC pathogenesis, acting through PAR-4 activation. Moreover, fecal supernatants from UC patients were able to trigger a permeability increase *in vitro* on mice colonic strips, which effect was blocked by the PAR-4-antagonist or the Cat-G inhibitor, but not influenced by the PAR-1 and PAR-2 antagonist. The synthetic PAR-4-AP, AYPGKF-NH_2, was able to reproduce this effect. This molecule has no effect on either PAR-1 or PAR-2 (44), and its effects are blocked by a PAR-4 antagonist (46). Additionally, fecal supernatants from UC patients with high serine-protease activity provoked an inflammation when infused intracolonically (*ic.)* in mice, demonstrated by the myeloperoxidase assay. This inflammatory effect was blocked by the Cat-G inhibitor, confirming that it is Cat-G mediated, possibly through the barrier impairment.

Abdominal pain and discomfort are among the most frequently reported gastrointestinal symptoms. IBD patients often mention these complaints, however, patients suffering from functional gastrointestinal disorders, like irritable bowel syndrome (IBS), may have similar symptoms. The diagnosis of IBS is established according to the Rome III. criteria, that is, recurrent abdominal pain or discomfort at least 3 days/month in the last 3 months associated with at least 2 of the followings: improvement with defecation and/or onset associated with a change in frequency and/or consistency of stool. These criteria must be fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis (58). Based on stool pattern, IBS has different subtypes: IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), mixed (IBS-M), when both diarrhea and constipation occur, and unclassified (IBS-U), when the abnormality of stool consistency is insufficient to be classified in the

three previous subtypes (58). Distension-induced rectal hyperalgesia and allodynia in IBS have been first described in 1973 (75), and since confirmed by others (15, 61, 95). In the following years similar studies found active UC patients hypersensitive to both noxious or non-noxious rectal distension, while most of the patients having a quiescent disease appeared normosensitive, except of those with a smaller rectal size (33, 74). However, later investigations have found that IBD patients do not have visceral hypersensitivity to distension (11, 22). Indeed, Crohn's disease patients presented a higher perceptual threshold of discomfort for slow ramp rectosigmoid distension and their skin conductance responses to aversive distension were greatly reduced compared to healthy subjects or IBS patients (11). Similarly, UC patients with quiescent disease or mild chronic rectal inflammation had higher discomfort thresholds in response to rectal distension following a noxious sigmoid stimulus compared with either IBS patients or healthy controls (22). In both studies distensions were conducted in the rectum, where CD patients had no inflammation, and UC patients had no or only mild inflammation. Intestinal inflammation is generally associated to increased visceral sensitivity, as mucosal immunal cells are able to release algogenic molecules in the proximity of nerve endings (10, 21, 31), and mediators released from non-immune cells have also been proposed to play a role in sensitizing afferent neurons (10, 12, 39). However, no inflammatory mediator has been found so far which can explain the rectal hyposensitivity observed in IBD patients.

It is known that PARs are also involved in the sensation of visceral pain. Intracolonic infusion of PAR-2-AP has been shown to induce colorectal hypersensitivity to distension in rats (26). Further, fecal supernatants from IBS-D patients containing a high level of serine-proteases elicits colorectal hypersensitivity to distension in mice and colonic paracellular permebility increase via PAR-2 activation (35). However, it is possible that other PARs have opposite effects, as intraplantar administration of a PAR-1 or a PAR-4-agonist provoked analgesia to both mechanical and thermal stimuli (6, 7). PAR-4 has been shown in the colonic epithelium (67), but also in dorsal root ganglion (DRG) neurons. Its activation on DRGs does not induce a calcium signal, but reduces the calcium signal evoked by KCl, suggesting that PAR-4 activation may inhibit the nociceptive signal in DRG neurons (7). Additionally, patch clamp recordings show that PAR-4 activation in DRG neurons projecting to the colon suppresses their excitability (50). PARs are also found on the surface of different immune cells throughout the gastrointestinal tract, where their activation may trigger the release of various chemokines and growth factors (85), interfering with nociceptive pathways. Specifically, PAR-4 is expressed by Kupffer cells, B-lymphocytes (78) and mast cells (63), and its activation affects leukocyte mobility (19, 62). Hence it is not clear whether the effect evoked by a PAR-4 agonist infused *ic.* to mice is mediated through a direct or indirect mechanism by activation of receptors located on colonic epithelial cells or immune cells favouring the release of

antinociceptive substances, or the agonist may act directly on nerve terminals. PAR-4 may be activated on the apical site of colonocytes, however, due to its small size (<15 kDa) PAR-4-AP may penetrate through tight junctions to reach mucosal nerve terminals. Moreover, in UC patients, luminal Cat-G content may step in contact with inflamed mucosa, where hyperalgesic neuromodulators are highly present, which may influence its effects. PAR-1 and PAR-4 activation are analgesic to thermal and mechanical stimuli on the rat paw in both basal and inflammatory conditions (6, 7). Further, PAR-4 activation has been shown to reverse PAR-2 or transient receptor potential vanilloid receptor-4 (TRPV-4) activation-induced colorectal hypersensitivity in mice (9).

2. AIMS

Based on this background, the aims of the studies summarized in the thesis were to evaluate the mechanism by which PAR-4 receptor and its activator, the neutrophil serine-protease Cat-G can play a role in the pathology of ulcerative colitis, and to explore the role of PAR-4 activation in visceral pain.

2.1 The aim of the human investigations

to compare the activity of Cat-G and serine protease activity in the feces of UC and IBS-D patients to that of controls.

2.2 The aims of the experiments on mice

1. to measure if fecal supernatants from UC patients change colonic paracellular permeability *in vivo*, and if this effect is linked to PAR-4 activation and Cat-G,
2. to test if fecal supernatants from UC patients are able to provoke colonic inflammation, and if this effect is mediated by PAR-4 activation and Cat-G,
3. to explore if the effects of fecal supernatants from UC patients on colonic paracellular permeability and inflammation are linked to myosin light chain kinase activation
4. to evaluate if fecal supernatants from UC patients affect colorectal mechanical sensitivity, and if this effect is mediated by PAR-4 and Cat-G,

5. to assess if the colorectal hypersensitivity provoked by fecal supernatants of IBS-D patients is linked to PAR-4 activation,
6. to localize the receptors participating in the effect of PAR-4-AP and Cat-G on visceral sensitivity,
7. to test the effect of colorectal PAR-4 activation on visceral sensitivity in two visceral hypersensitivity models.

3. MATERIALS AND METHODS

3.1. Human investigations

3.1.1.Fecal sample collection

Healthy subjects (male/female: 4/7; mean age: 44 yrs), IBS-D (male/female: 3/8, mean age: 53 yrs) and UC (male/female: 13/10; mean age: 44 yrs) patients were recruited in First Department of Medicine, Szeged, Hungary. The study protocol was approved by the Ethical Committee of the University of Szeged. All subjects provided written and informed consent to participate. IBS-D patients fulfilled the Rome III criteria. Organic gastrointestinal disorders were excluded in these patients by detailed blood and stool analyses, serologic assays for celiac disease, lactose-hydrogen breath test and colonoscopy. UC patients were evaluated clinically and endoscopically. According to the protocol of Chang et al. (22), we selected UC patients presenting either a quiescent or mildly active UC with mean clinical activity index, CAI: 9.4±1.7 (Rachmilewitz, (73)). Fecal samples from healthy subjects, UC and IBS-D patients were collected and frozen within one hour after defecation in the First Department of Medicine, Szeged, Hungary.

3.1.2. Preparations of fecal supernatants

The samples were kept in -20ºC and transported in dry ice to the Institut National de la Recherche Agronomique, Toulouse, France. On arrival, the samples were thawed at 4ºC. 0.5 g of each sample was diluted, mixed and homogenised in 4 mL of 0.9 % NaCl solution. After centrifugation (10 min, 4500 rpm, 4ºC), pellets were discarded and supernatants were filtered by 0.8 μm-sized syringe filters.

3.1.3. Measurement of fecal total protease activity

Supernatants of fecal homogenates (25 μl) were incubated with 1 mL of reaction buffer (0.15 M NaCl and 20 mM Tris-HCl, pH 8.3) and 1 mL of 0.5 % (w/v) azocasein (Sigma-Aldrich, Saint-Louis, Missouri, USA) at 40ºC. The reaction was stopped after 20 min with 1 mL of 10 % (v/v) trichloracetic acid (TCA, Sigma). Following centrifugation, absorption of the clear supernatant was measured at 366 nm. Enzymatic activities of the supernatants were normalised to protein content, assessed with BCA-200 Protein Assay Kit (Pierce, Rockford, IL) using bovine albumin as standard. The effect of serine-protease inhibitors was tested by preincubating the supernatants with a mixture of aprotinin (Sigma, final cc. 0.22 mg/mL) and soybean trypsin inhibitor (Sigma, SBTI, final cc. 1.70

mg/mL) for 30 min on 4ºC. UC samples were also incubated with Cat-G inhibitor (Calbiochem, Darmstadt, Germany; 10:1 from 1 mM solution in dimethyl-sulphoxide, DMSO) on ice for 30 min prior to measurement of protease activity. Results were expressed in trypsin units of activity/mg protein. Our aims were to test the effect of serine-protease activity on visceral sensitivity, therefore we selected the samples with a serine-protease activity superior than 1310 U/mg protein (which was the highest activity among healthy subjects). In the samples tested, no significant difference has been observed in serine-protease activity (SPA) measured in male *vs* female IBS-D and UC patients (P=0.77, P=0.49, respectively).

3.1.4. Measurement of fecal Cat-G activity

Cat-G activity in the fecal supernatant was measured by an enzymatic assay using N-succinyl-Ala-Ala-Pro-Phe p-Nitroanilide (Sigma) as a more selective substrate. 1600 µl of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) NaOH buffer (100 mM, pH 7.5), 200 µl substrate (20 mM) and 100 µl of fecal supernatant were mixed with fecal supernatant from UC, IBS-D patients, healthy subjects or UC supernatant previously incubated with the mixture of aprotinin and SBTI or Cat-G inhibitor, then enzymatic activity was measured at 410 nm for 5 min on 37ºC.

3.2. Animal experiments

3.2.1. Animals

Male 6-8 weeks old BALB/cBy mice were used for the experiments on colonic permeability and inflammation. However, visceral sensitivity experiments were performed on C57BL/6J wild type mice, except from BALB/cBy Prkdc ^{scid} (severe combined immunodeficient, SCID) mice and their BALB/cBy controls (Janvier, Le Genest St-Isle, France), as C57BL/6J mice were found ideal candidates for assessing visceral pain sensation in previous studies performed in our laboratory (35). The animals were kept in polypropylene cages in a temperature-controlled room with 12-hour dark-light cycles, water and standard pellets (Harlan Teklad, Oxon, UK) were provided *ad libitum*. All experimental procedures were approved by the local Institutional Animal Care and Use Committee.

3.2.2. Effects of PAR-4 activation on colonic permeability and inflammation *in vivo*

3.2.2.1. Intracolonic infusions

BALB/cBy mice were fasted for 24 hours and then placed in plastic tubes (3 cm in diameter, 9.5 cm in length) for 60 minutes in order to empty their bowel contents, thus reducing the risk of perforation during catheter insertion. Mice were anesthetized with sodium pentobarbital (5 mg/kg, Ceva, Libourne, France) and a polyethylene perfusion catheter was inserted into the distal colon ending 3.5 cm from the anus. After recovery from anesthesia, animals received intracolonically (*ic.*), either 1) 0.3 mL of fecal supernatants of UC patients (proteolytic activity: 2193 ± 430 U/mg of protein); 2) 0.3 mL of fecal supernatants of healthy subjects (proteolytic activity: 698 ± 128 U/mg of protein); 3) 0.3 mL of fecal supernatants of UC patients previously incubated with specific cathepsin-G inhibitor (0.2 mM, Calbiochem, Pessac, France) for 30 minutes on ice; 4) PAR-4 antagonist (P4-pal10, pepducin, NeoMPS, Strasbourg, France) intraperitoneally (*ip.*) or its vehicle 3 times (3 x 0.25 mg/kg) prior to the *ic.* administration of 0.3 mL of fecal supernatants of UC patients vehicle; 5) PAR4 agonist peptide (PAR-4-AP, Ala-Tyr-Pro-Gly-Lys-Phe-NH2, 100 µg/mice, Sigma, St. Quentin Fallavier, France); 6) Cat-G (0.025 U, Sigma); 7) P4-pal10 pepducin *ip.* or its vehicle 3 times (3 x 0.25 mg/kg) prior to the *ic.* administration of PAR4 agonist (100 µg/mice); 8) P4-pal10 pepducin *ip.* or its vehicle 3 times (3 x 0.25 mg/kg) prior to the *ic.* administration of Cat-G (0.025 U); 9) 2 mg/kg of the MLCK blocker ML-7 *ip.* (dissolved in 10% ethanol) or its vehicle 3 times prior to the *ic.* administration of Cat-G or its vehicle; 10) vehicle. All reagents were infused *ic.* with a perfusion rate of 170 µL/h.

3.2.2.2. *In vivo* permeability studies

Two hours after *ic.* infusions mice received by gavage 200 µl of ^{51}Cr-ethylenediamine tetra-acetic acid (^{51}Cr–EDTA, 0.35 µCi diluted in water, Perkin Elmer Life Science, Courtaboeuf, France). Mice were then placed in metabolic cages and urine was collected for 6 hours. Total radioactivity found in urine was measured with a gamma counter (Cobra II; Packard, Meriden, CT). Permeability to ^{51}Cr–EDTA was expressed as a percentage of the total radioactivity administered.

3.2.2.3. Myeloperoxidase activity assay

The activity of myeloperoxidase (MPO) in mice distal colon was measured as described earlier (16). Briefly, samples of distal colon (3 cm) were suspended in a potassium phosphate buffer (50 mM, pH 6.0) and homogenized on ice. Three cycles of freeze-thaw were undertaken. Suspensions were then centrifuged at 10 000 g for 15 min at 4°C. Supernatants were discarder and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (HTAB 0.5% w/v, in 50 mM potassium phosphate buffer, pH 6.0). These suspensions were sonicated on ice, and centrifuged

again at 10 000 g for 15 min at 4°C. The supernatants obtained were diluted in potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL of O-dianisidin dihydrochloride and 0.0005% of hydrogen peroxide. Myeloperoxidase from human neutrophils (0.1 units per 100 µL) was used as a standard. The kinetic changes in absorbance at 450 nm, every 10 s over 2 min, were recorded with a spectrophotometer. One unit of MPO was defined as the quantity of MPO degrading 1 µmol of hydrogen peroxide/min/mL at 25°C. Protein concentration was determined with a commercial kit using a modified method of Lowry (protein Quantitation Kit, Interchim, Montluçon, France). MPO activity was expressed as units per gram of protein.

3.2.2.4. Immunohistochemistry for phosphorylated MLC (pMLC)

For phosphorylated MLC (pMLC) immunolabeling, samples (distal colon) were fixed in buffered paraformaldehyde (4%), incubated in 30% sucrose (24 h, 4°C), embedded (Tissue Tek medium), and frozen in isopentane at -45°C. Cryostat sections (7 µm) were fixed with acetone (10 min, -20°C), hydrated in phosphate-buffered saline (PBS), permeabilized with PBS-0.5% Triton X-100, and incubated in blocking solution (PBS containing 1% bovine serum albumin). For pMLC staining, samples were then incubated with goat anti-pMLC antibodies (1/100, Santa Cruz Biotechnologies, Santa Cruz, CA) followed by incubation with biotin-conjugated IgG donkey antigoat antibody (1/1000, Interchim). Sections were rinsed in NaHCO3 (0.1 M, pH 8.2) and incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (1/500) diluted in the same solution. All sections were mounted in Vectashield HardSet Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and examined under a Nikon 90i fluorescent microscope.

3.2.2.5. Western blotting for phosphorylated MLC (pMLC)

Samples were homogenized on ice in an extraction buffer (24 mM Tris buffer, pH 7.4 containing 137 mM NaCl, 2.68 mM KCl, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) Igepal CA630 (octylphenoxypolyethoxyethanol), 0.5% (w/v) sodium deoxycholate, supplemented with protease inhibitor cocktail 1 mM [Roche] and phosphatase inhibitor cocktails I and II [Sigma]). For analysis, 15 µg of proteins were loaded onto a 15% SDS-polyacrylamide gel and transferred to 0.22 µm nitrocellulose membranes at 80 V for 1 hour. Nonspecific binding sites were blocked for 1 hour at room temperature in a blocking buffer (ScienceTec, Les Ulis, France). Membranes were then incubated with rabbit anti-pMLC 1/200 (Biosource, Cergy-Pontoise, France), rabbit anti-MLC 1/200 (Cell Signaling Technology, Danvers, MA), or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) 1/1000 antibodies (Cell Signaling Technology), overnight at 4°C.

After washing, membranes were incubated with fluorescent CF770 antirabbit 1/10,000 antibody (Biotium, Hayward, CA) with gentle rocking and protected from light. Finally, they were scanned on infrared imaging system Odyssey (Li-Cor, Lincoln, NE).

3.2.3. Experiments on visceral pain

3.2.3.1. Measurement of visceral sensitivity in mice

All visceral pain experiments were performed on C57Bl.6/J mice, except from the study on SCID mice. Via cutaneous abdominal incision two nickel-chrome electrodes were implanted in the abdominal external oblique muscle and the third in the abdominal skin following xylasine-ketamine anesthesia subcutaneously (1.2 mg, respectively). The electrodes were drawn to the back of the neck subcutaneously and protected by a plastic tube. On the 4-7th postoperative days colorectal distensions were performed as painful stimuli in order to evoke abdominal electromyographic response as a sign of visceral sensitivity. The animals were placed in a plastic tunnel 1 hour before the beginning of the experiments in order to empty their bowel contents. After 1 hour of habituation period, mice were lightly anaesthetised by sodium pentobarbital (1 mg, ip.), and polyethylene perfusion and distension catheters (Fogarty catheter for artherial embolectomy, 4F, balloon length: 1.1 cm, Edwards Lifesciences, Nijmegen, The Netherlands) were inserted into the colon (the tip of the catheters were 3.5 and 2.5 cm from the anus, respectively), and mice were reintroduced into the plastic tunnels. Colorectal distension (CRD) procedure started 60 min after the colorectal infusions had finished, with volumes progressively increasing in 0.02 mL steps from 0 to 0.12 mL, by injecting physiological saline to the balloon with a Hamilton syringe (500 µL, Hamilton Company, Bonaduz, Switzerland), each step lasting 10 s with 5 min non-distension periods in-between. During the distension periods the abdominal muscle electrical activity was recorded and analyzed with Powerlab Chart 5 program from AD instruments (Colorado Springs, United States). Basal electromyographic (EMG) activity was subtracted from the EMG activity registered during the periods of distension.

3.2.3.2. Colorectal infusions

Colorectal infusions were performed as described above. Mice were infused ic. with either 1) 0.3 mL fecal supernatant from healthy subjects, UC or IBS-D patients. 2) P4-pal10 pepducin ip. or its vehicle 3 times (3 x 0.25 mg/kg, 30 min before, 60 and 120 min after the beginning of infusion)

combined with the ic. administration of 0.3 mL of fecal supernatants of healthy subjects, UC or IBS-D patients or vehicle; 3) 100 μg of a PAR-4-AP (, 0.667 mg/mL 0.9 % NaCl) or its vehicle (0.9 % NaCl) with or without PAR-4-antagonist; 4) 0.025 unit (UN) of Cat-G (Sigma, 0.167 UN/mL in 0.9% NaCl) ic. or its vehicle (0.9 % NaCl); 5) UC fecal supernatants previously incubated on ice for 30 min with specific cathepsin-G inhibitor (Cat-G inhibitor, 10:1 from 1 mM solution in dimethyl-sulphoxide, DMSO) or with a mixture of aprotinin and SBTI (aprotinin: final cc. 0.22 mg/mL; SBTI: final cc. 1.70 mg/mL) for 30 min prior to infusion. Incubation with DMSO was performed as control for Cat-G inhibitor. 6) 0.3 mL fecal supernatants from IBS-D patients with or without the combination of Cat-G (total amount of Cat-G: 0.025 UN; 0.0083 UN administered as a pretreatment). 7) PAR-4-AP (100 μg in 0.15 mL 0.9% NaCl) or its vehicle in ic. bolus injection or in ic. infusion. 8) 2,4,6-triaminopyrimidine (TAP, Sigma; total dose: 30 μmol/mouse in 150 μl 0.9% NaCl) ic. 1 hour preceding PAR-4-AP ic. infusion (as previously described), followed by a parallel administration.

3.2.3.3. Experiments on SCID mice

Male SCID mice and their BALB/cBy controls were operated as C57BL/6J mice, and on the 4th postoperative day mice received ic. infusion of 100 μg PAR-4-AP or vehicle as described above. Visceral pain measurements were started 1 hour following the end of infusion.

3.2.3.4. Visceral hypersensitivity models

3.2.3.4.1. Water avoidance stress model

Modification of a previously described protocol has been used (56). Briefly, C57BL/6J mice were placed on a 3x3 cm platform in a 40x40 cm size pool filled with tapwater during one hour on four consecutive days. The animals who fell into the water were gently dried with a towel and placed back to the platform. Prior to CRD, fecal samples of mice were collected for Cat-G activity measurement and Cat-G activity in the fecal supernatant was determined as described above.

3.2.3.4.2. Colonic microinflammation

Low grade colonic inflammation was provoked by *ic.* administration (tip of the catheter 3.5 cm from the anus) of a low dose of trinitrobenzene sulfonic acid (TNBS, Sigma, 20 mg kg^{-1} mice) in 30% ethanol or 0.9% NaCl in C57BL/6J mice on the 4th postoperative day, as described earlier (32). Visceral sensitivity measurements were performed 72 h after the administration of TNBS. Prior to

CRD, fecal samples of mice were collected for Cat-G activity measurement and Cat-G activity in the fecal supernatant was determined as described above.

3.2.3.4.3. Intracolonic infusions

Separate groups of WAS and TNBS mice received *ic.* infusion of 1) 100 µg PAR-4-AP or its vehicle; 2) 0.025 UN of Cat-G (0.167 UN mL^{-1} in 0.9% NaCl) or its vehicle; 3) 0.75 mg kg^{-1} PAR-4 antagonist (P4pal-10, in 0.15 mL 0.9% NaCl) or its vehicle *ip.*.

3.2.3.5. Fos immunohistochemistry

Mice received *ic.* PAR-4-AP infusion (100 µg in 0.15 mL 0.9% NaCl) or its vehicle, 0.9% NaCl and one hour later underwent the CRD protocol as described above. A group of mice without treatment and distension served as naive controls. One hour after the completion of the CRD, mice were deeply anesthetised with xylazine-ketamine (both 2 mg *ip.*) and perfused transcardially with 50 mL physiologic saline followed by 50 mL of 4% paraformaldehyde. After fixation, lumbosacral segments (L5-S1) of the spinal cord were dissected and removed, postfixed at +4°C in 4% buffered paraformaldehyde, incubated in 30% sucrose (24 h, +4°C), embedded (Tissue Tek medium) and frozen in isopentane at -45°C. Frozen serial sections (35 µm) were collected in phosphate-buffered saline (PBS), then rinsed twice. Sections were stained for Fos-like immunoreactivity using biotin-avidin-peroxidase complex. Sections were incubated at room temperature in a blocking solution of 2% normal goat serum in PBS with 0.25% Triton X-100 for 30 minutes and then incubated overnight at 4°C with rabbit polyclonal Fos antibody diluted in blocking solution (1:10000; Ab-5, AbCys, Paris) for 24 hours at 4 °C. The incubated sections were washed twice and incubated with biotinylated goat anti-rabbit secondary antibody, diluted 1:1000 in blocking solution, and then incubated with the avidin-biotin complex (Vectastain Elite kit; Vector Laboratories, Paris, France). Peroxidase activity was revealed using diaminobenzidine as chromogene (DAB substrate kit, Vector Laboratories, France). The sections were then mounted on gelatin-coated slides, deshydrated, and coverslipped with DePex. The presence of Fos immunoreactivity was detected as a dark brown reaction product in cell nuclei under a light microscope (90i Nikon, Nikon France, Champigny-sur-Marne, France). The number of cells containing Fos immunoreactivity was counted in the laminae I-II and the area surrounding the central canal (area X) bilaterally in 16 consecutive sections of the lumbosacral segment of the spinal cord (L5-S1) using Lucia G4.8 software.

3.3. Statistical analysis

All data are expressed as means±SEM. Densitometry analysis was conducted using Image J software (NIH, Bethesda, MD). For statistical analysis Graph Pad Prism 4.0 (GraphPad, San Diego, CA) was used. Comparison between two groups was performed by Student's paired t-test. Multiple comparisons within groups were performed by one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was accepted at $P<0.05$.

4. RESULTS

4.1. Human investigations

4.1.1. Protease and Cat-G activity in fecal supernatants

The total protease activity of fecal supernatants from IBS-D and UC patients was more than 2.5 fold higher than that measured in healthy subjects ($P<0.05$, $P<0.01$, respectively). Preincubation with the mixture of the serine protease inhibitors, aprotinin and SBTI, decreased the total protease activity of UC supernatants close to the fecal protease activity of healthy subjects ($P<0.01$). The Cat-G inhibitor decreased protease activity by 13% ($P>0.05$) (Table 1.). Cat-G activity is significantly elevated in UC supernatant compared to either healthy subjects or IBS-D supernatant ($P<0.001$, $P<0.001$). However, no significant difference was observed in the Cat-G activity of IBS-D *vs* healthy subjects supernatant. ($P>0.05$). The mixture of serine-protease inhibitors, aprotinin and SBTI, suppressed Cat-G activity in the UC supernatant ($P<0.001$). Incubation with a Cat-G inhibitor decreased the activity by 43% ($P<0.01$; Table. 1).

supernatant	Healthy subject	IBS-D	UC	UC+aprotinin/SBTI	UC with Cat-G inhibitor
PA (trypsin U/mg protein)	521 ± 79	$1832 \pm 262^{*}$	$1801 \pm 106^{**}$	$687 \pm 100^{\#\#}$	1572 ± 202
Cat-G activity (U/min/g protein)	1.85 ± 0.4	3.61 ± 1.6	$28.34 \pm 3.04^{***, \S\S\S}$	$0.97 \pm 0.38^{\#\#\#}$	$16.14 \pm 2.02^{\#\#}$

Table 1. Protease (PA) and cathepsin G (Cat-G) activities in fecal supernatants from healthy subjects, diarrhea predominant irritable bowel syndrome (IBS-D) and ulcerative colitis (UC) patients. (*P<0.05, **P<0.01, ***P<0.001 vs healthy subjects; # # P<0.01, # # # P<0.001 vs UC supernatant; §§§P<0.001, vs IBS-D supernatant)

4.2. Animal experiments

4.2.1. Effects of PAR-4 activation on colonic permeability and inflammation *in vivo*

4.2.1.1. Colonic inflammation: myeloperoxidase (MPO) activity

Two hours after intracolonic infusion of fecal supernatant from UC patients (110.2 ± 16.7 U MPO/g protein), the level of MPO found in colonic tissue was significantly increased compared with fecal supernatants from healthy subjects (65.5 ± 12.1 U MPO/g protein, $P < 0.05$) and saline (45.4 ± 6.0 U MPO/g protein, $P < 0.001$) (Fig. 1A). Moreover, Cat-G inhibitor was able to abolish the increase in MPO activity, observed after UC fecal supernatants infusion (67.0 ± 16.7 U MPO/g protein, $P < 0.05$, Fig. 1A). At the concentration tested (0.75 mg/kg) pepducin P4pal-10 abolished the

effect of UC fecal supernatants (64.4 ± 13.5 U MPO/g protein, $P < 0.05$) on MPO activity (Fig. 1A). Intracolonic infusion of Cat-G and PAR4-AP also increased MPO activity in comparison with vehicle (respectively 303.0 ± 43.5 U MPO/g protein, $P < 0.001$ and 184.1 ± 30.3, $P < 0.05$) (Fig. 1B). Further, the increase in MPO activity triggered by Cat-G was significantly reduced by ML-7 (274.0 ± 34.0 U MPO/g protein versus 190.5 ± 15.1, $P < 0.05$, Fig. 1B).

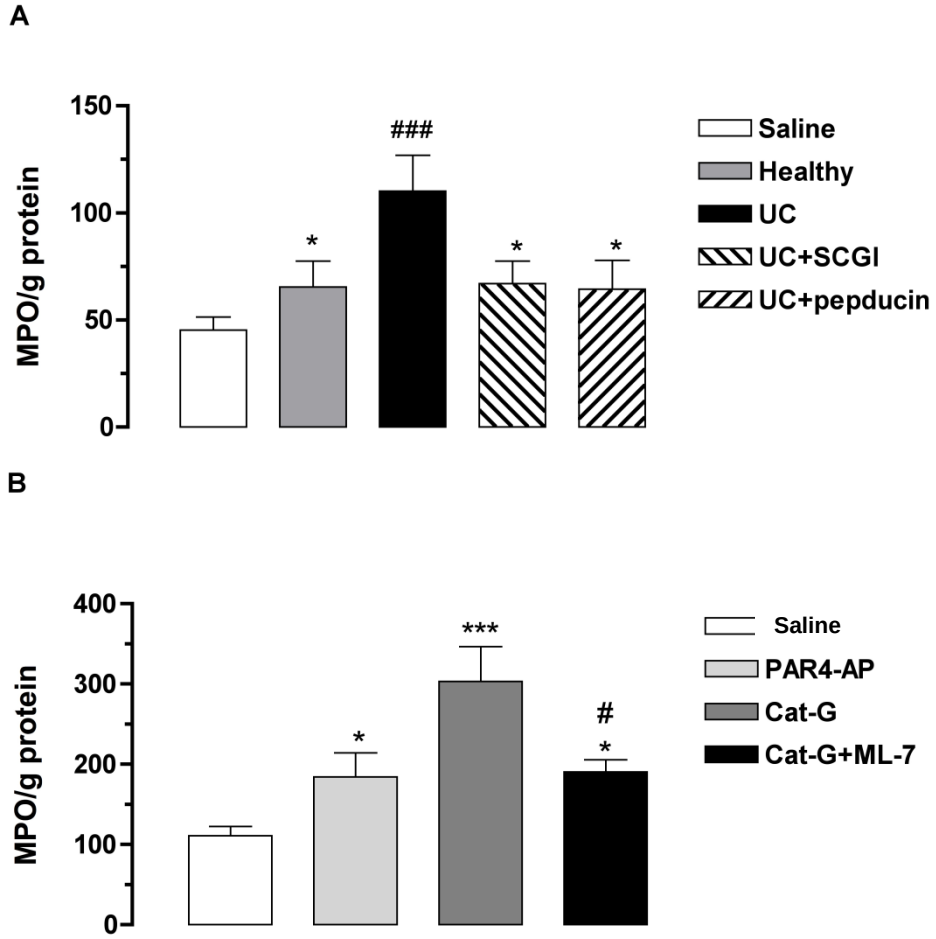


Fig. 1. The effect of *ic*. infusion of **A)** vehicle (saline), fecal supernatants from healthy subjects, ulcerative colitis (UC) patients, UC supernatants + Catepsin-G inhibitor (SCGI) or UC supernatants combined with pepducin P4-pal10 (*ip.*), **B)** vehicle, PAR-4 activating peptide (PAR-4-AP), Cathepsin-G (Cat-G), (or Cat-G combined with ML-7 (*ip.*) on colonic myeloperoxidase (MPO) activity. Data are expressed as means±SEM (n=6/7), $^{###}P<0.001$ compared with saline and $*P<0.05$ compared with UC (A) and $*P<0.05$ and $^{***}P<0.001$ compared with saline and $^{\#}P<0.05$ compared with Cat-G (B).

4.2.1.2. Colonic paracellular permeability

Ic. infusion of fecal supernatants from healthy subjects did not significantly affect colonic paracellular permeability compared with vehicle, saline (2.2±0.3 % of ^{51}Cr-EDTA *vs.* 1.8±0.2). In contrast, *ic.* infusion of fecal supernatants from UC patients significantly increased by 68.8%

(3.7±0.5 % of ^{51}Cr-EDTA) the passage ^{51}Cr-EDTA compared with vehicle (P<0.01) and with supernatants from healthy subjects (P<0.05) (Fig. 2A). Moreover, pepducin-P4-pal10 abolished (2.0±0.2 % of ^{51}Cr-EDTA P<0.05 *vs.* UC; NS *vs.* saline and healthy) the effect of UC fecal supernatants (Fig. 2A). *Ic.* delivery of PAR-4 activating peptide and Cat-G increased the paracellular permeability in comparison with vehicle infusion (2.5±0.3 %, P<0.05 and 3.6±0.6, P<0.01 *vs.* 1.8±0.2). Here again, at the concentration tested (0.75 mg/kg), pepducin P4pal-10 abolished the effect of PAR-4-AP on paracellular permeability (Fig. 2B). Finally, pretreatment of Balb/c mice with the MLCK inhibitor, ML-7, reduced the increased paracellular permeability induced by Cat-G by 92.3% (1.3±0.1% of ^{51}Cr-EDTA *vs.* 2.5±0.2, P<0.01) (Fig. 2B).

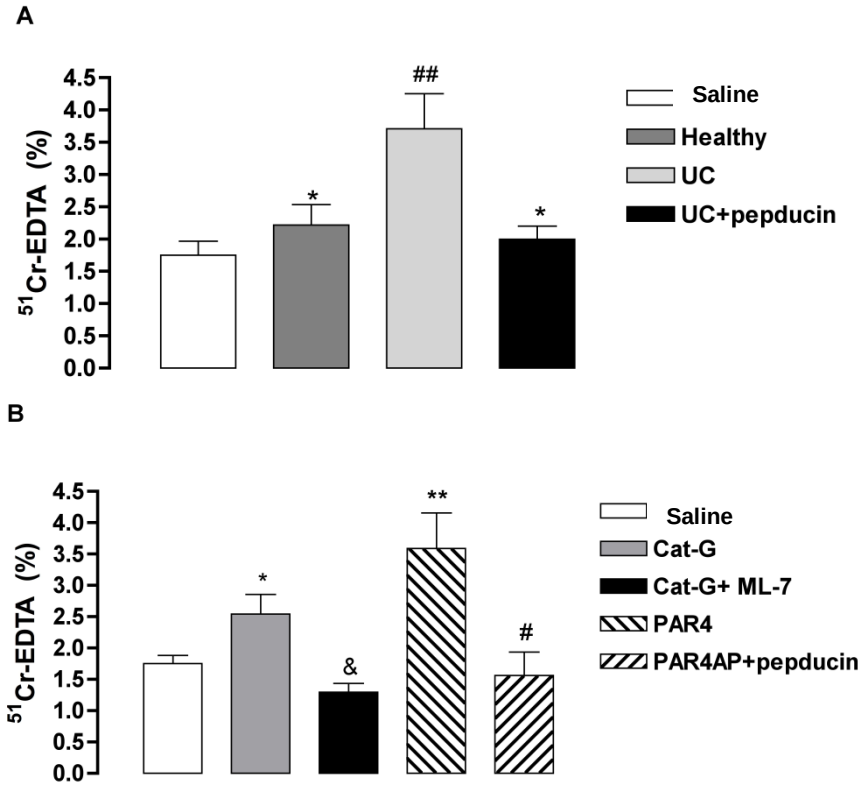


Fig. 2. The effect of *ic.* infusion of **A)** saline, fecal supernatants from healthy subjects, ulcerative colitis (UC) patients and UC supernatants in combination with pepducin (*ip.*), **B)** saline, Cathepsin-G (Cat-G), Cat-G + ML-7 (*ip.*), PAR-4 activating peptide (PAR-4-AP), PAR-4 combined with pepducin (*ip.*) on gut paracellular permeability. Data are expressed as means±SEM (n=6/7), $^{\#\#}$P<0.01 compared with saline and *P<0.05 compared with UC (A) and *P<0.05 and **P<0.01 compared with saline, $^{\#}$P<0.05 compared with PAR-4 and $^{\&}$P<0.05 compared with Cat-G (B).

4.2.1.3. Immunohistochemistry and Western blotting of phosphorilated MLC (pMLC)

Two hours after *ic.* infusion with fecal supernatants from UC patients an intense and diffuse labeling of pMLC was observed in epithelial cells similar to that observed after infusion of the PAR-

4 agonist or Cat-G (Fig. 3A). Western blotting confirmed these findings. We observed a higher level of pMLC in animals receiving UC fecal supernatant infusion (n = 3) compared with healthy supernatants or saline (n = 3) (Fig. 3B,C). On the contrary, administration of fecal supernatants from healthy subjects or vehicle did not modify pMLC labeling under basal conditions (Fig. 3B,C).

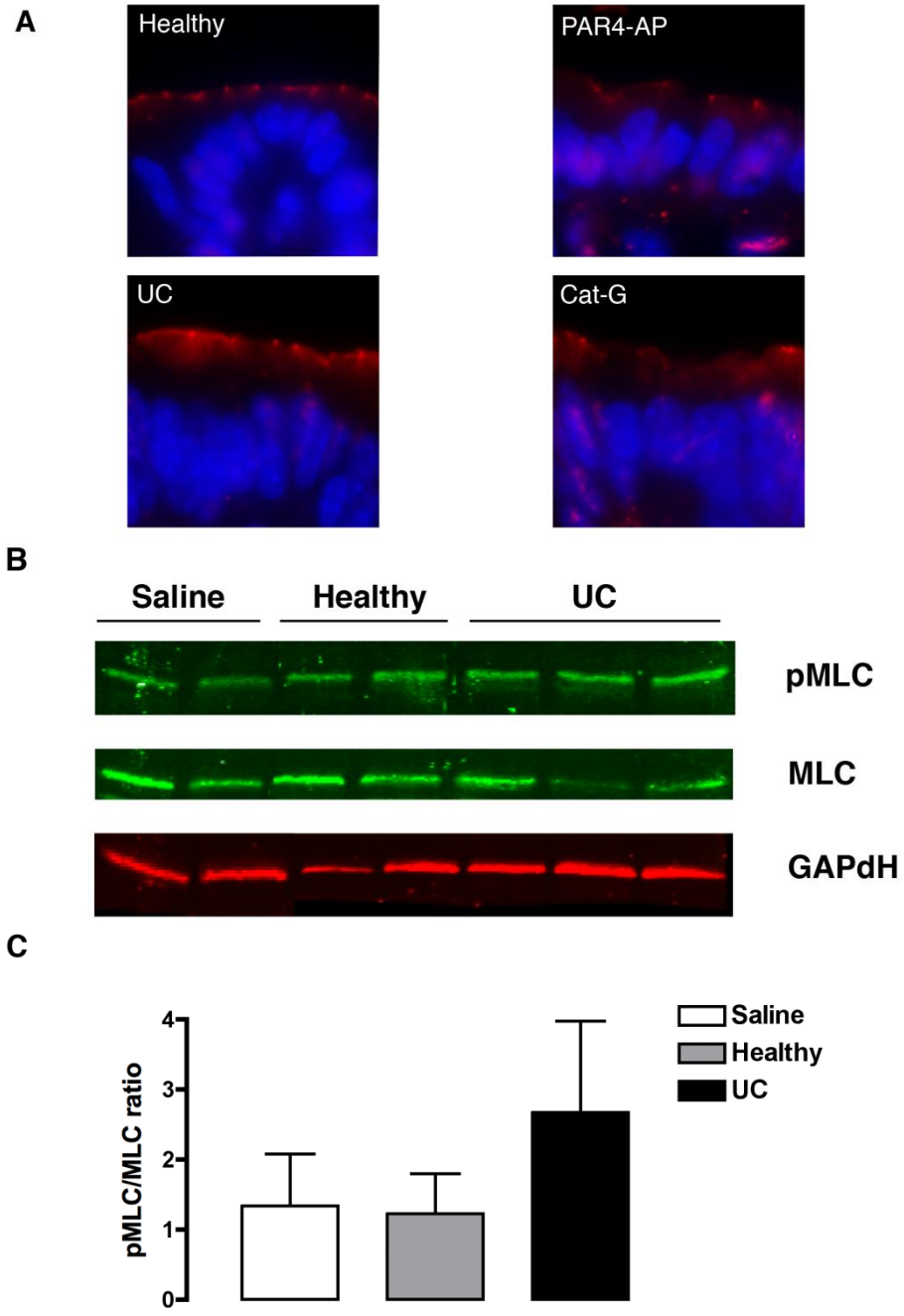


Fig. 3. A) Immunolabeling of phosphorylated myosin light chain (pMLC, red) and nuclear labeling with 4'0,6-diamidino-2-phenylindole (DAPI, blue) in enterocytes 2 hours after *ic.* infusion with fecal supernatants from healthy controls, ulcerative colitis (UC) fecal supernatants, Cathepsin-G (Cat-G), and PAR-4 activating peptide (PAR-4-AP). **B)** Western blotting experiments for pMLC, myosin light chain (MLC), and GAPdH in mouse colonic mucosa after infusion with saline, healthy, or UC supernatants. **C)** Quantitative analysis of Western blots.

4.2.2. Visceral sensitivity in mice

4.2.2.1. Effects of IBS-D, UC or healthy subjects supernatant infusions on visceral sensitivity

Ic. infusion of fecal supernatants from healthy subjects did not change visceral sensitivity compared to vehicle infusion (Fig. 4.). However, fecal supernatant from IBS-D patients significantly increased the abdominal muscular EMG response in mice by 860%, 167%, 59% and 21% at the steps of 0.02, 0.04, 0.06 and 0.08 mL, respectively, compared to mice treated with fecal supernatant from healthy subjects ($P<0.05$, $P<0.05$, $P<0.001$, $P<0.05$) (Fig. 4.). In contrast, colorectal instillation with fecal supernatant from UC patients resulted in a hyposensitivity illustrated by a significant decrease of the intensity of abdominal muscle contractions of mice by 72%, 49% and 35% at the distension volumes of 0.04, 0.06 and 0.08 mL, respectively, compared to animals treated with healthy supernatant ($P<0.01$, $P<0.05$, $P<0.05$, respectively) (Fig. 4.).

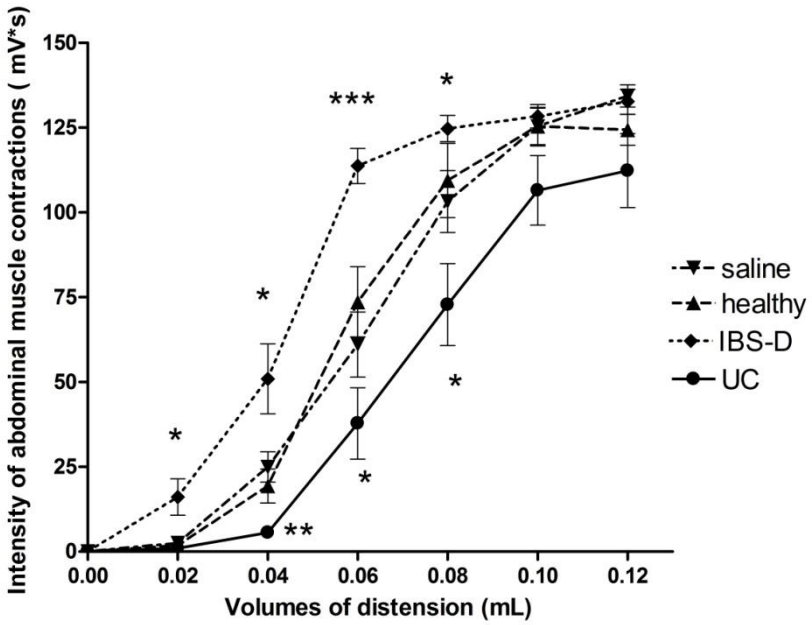


Fig. 4. Effects of healthy vs ulcerative colitis (UC) and diarrhea-predominant irritable bowel syndrome (IBS-D) fecal supernatant compared to colorectal infusion of saline on visceral sensitivity of mice. Each line represents the mean EMG response of all animals tested in the same group (infused with healthy: n=23, IBS-D: n=30, UC: n=38, *P<0.05, *P<0.05, ***P<0.001).

4.2.2.2. Effects of IBS-D, healthy or UC supernatant + PAR-4 antagonist infusions on visceral sensitivity

Ip. treatment with the PAR-4 antagonist pepducin did not modify the visceral sensitivity after *ic.* infusion of saline or healthy fecal supernatants ($P>0.05$, Fig. 5.A). Further, the PAR-4 antagonist

did not modify the hypersensivity provoked by *ic.* infusion of IBS-D fecal supernatants (Fig. 5B). In contrast, PAR-4 antagonist pepducin treatment before and during UC fecal supernatant infusion did not restore normal sensitivity, but exacerbated visceral sensitivity by 478%, 140% and 53% at 0.04, 0.06 and 0.08 mL distension volumes, respectively, in comparison to UC fecal supernatant infusion alone, causing a hypersensitivity similarly to IBS-D supernatant infusion (P<0.001, P<0.001, P<0.01, respectively) (Fig. 5.B).

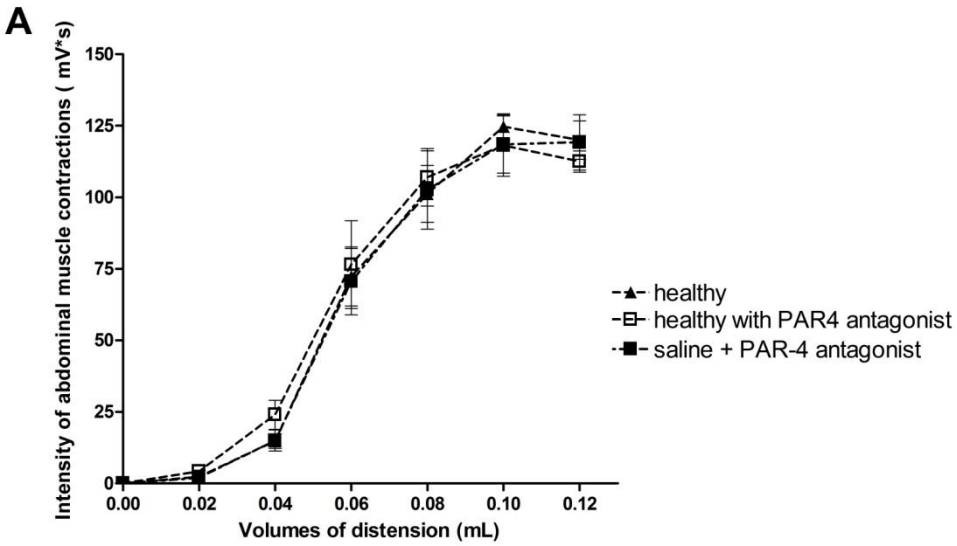


Fig. 5. A) Effects of PAR-4 antagonist in combination with *ic.* infusion of saline or fecal supernatant from healthy subjects.

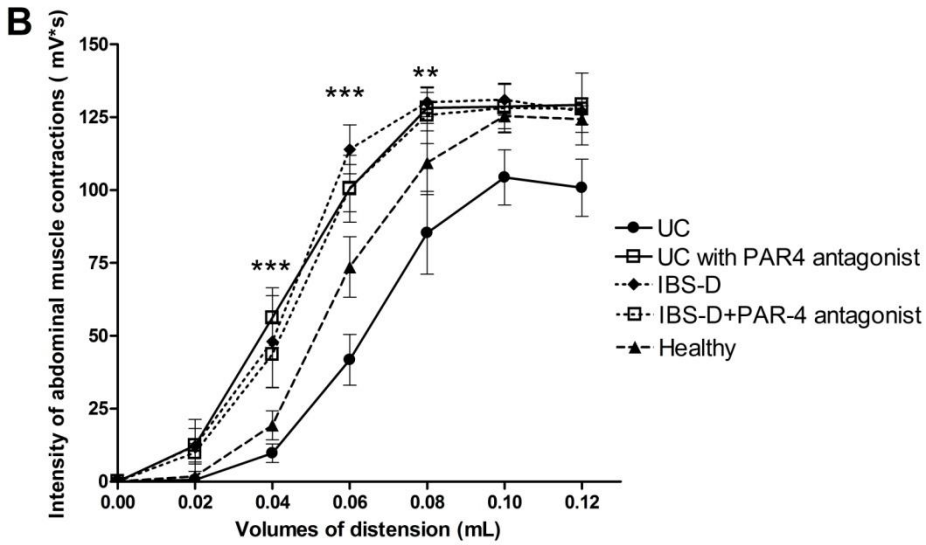
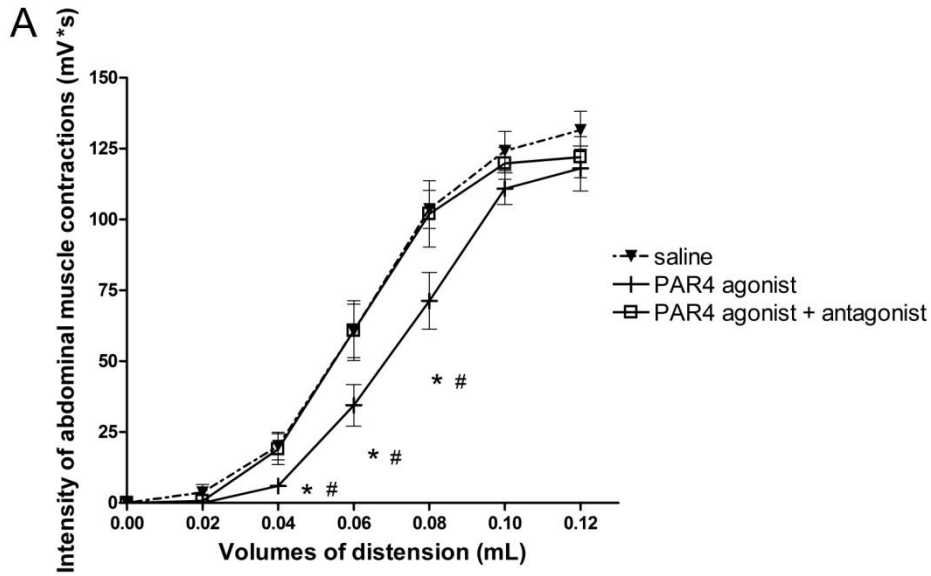


Fig. 5. B) Effects of PAR-4 antagonist in combination with *ic.* infusion of ulcerative colitis (UC) or diarrhea-predominant irritable bowel syndrome (IBS-D) fecal supernatants on visceral sensitivity of mice. (UC vs UC supernatant infusion + PAR-4 antagonist injection from the same patients; ***P<0.001, ***P<0.001, **P<0.01, respectively).

4.2.2.3. Effects of PAR-4 activating peptide and Cat-G infusions on visceral sensitivity

Ic. treatment of mice with the PAR-4-AP AYPGKF-NH_2 decreased abdominal EMG response to colorectal distension significantly by 70%, 43% and 25% compared to its vehicle, 0.9 % NaCl solution at the distension volumes of 0.04, 0.06 and 0.08 mL ($P<0.05$) (Fig. 6.A). The effect of PAR-4-AP was blocked by the *ip.* treatment of PAR-4 antagonist particularly at the distension volumes of 0.04, 0.06 and 0.08 mL ($P<0.05$) (Fig. 6.A). I*c.* infusion of Cat-G solution significantly decreased the abdominal muscle response to rectal distension for all the volumes from 0.04 to 0.12 mL compared to 0.9% NaCl infusion, particularly for 0.04 and 0.08 mL, by 73% and 40%, respectively ($P<0.01$, $P<0.02$) (Fig. 6.B). Combination of the supernatant from IBS-D patients with the Cat-G significantly inhibited the pronociceptive effect of IBS-D supernatant at the distension volumes from 0.04 to 0.1 mL ($P<0.05$, $P<0.001$, $P<0.01$, $P<0.05$, respectively) (Fig. 6.B).



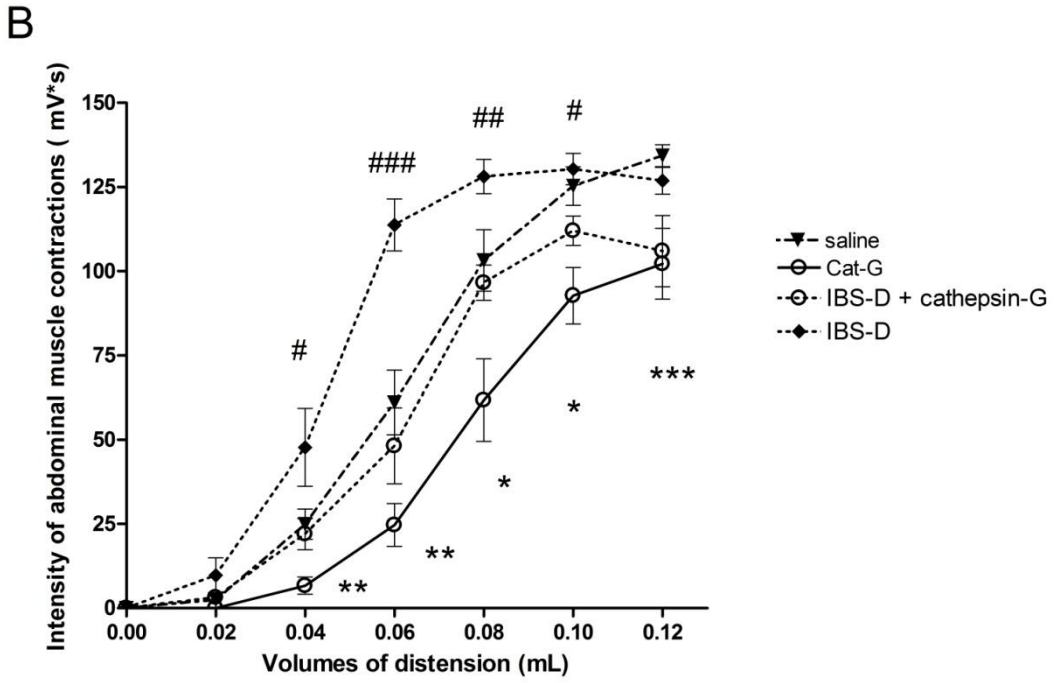


Fig. 6. Effects of PAR-4 agonist or cathepsin G colorectal infusion on visceral sensitivity of **A, B)** naive or **B)** IBS-D fecal supernatant-infused mice. A) n=10, saline vs PAR-4-AP, *P<0.05; PAR-4-AP vs PAR-4-AP + PAR-4-antagonist, $^{\#}$P<0.05. B) saline vs Cat-G, *P<0.05, **P<0.01, ***P<0.001.; IBS-D supernatant infusion from 4 patients, each tested on 3 mice, n=12, vs. IBS-D supernatant infusion from the same patients, each tested on 3 mice, n=12, #P<0.05, ##P<0.01, ###P<0.001.

4.2.2.4. Effects of Cat-G inhibitor or serine-protease inhibitors on visceral sensitivity

Ic. infusion of UC supernatant previously incubated with the Cat-G inhibitor resulted in a significant elevation of abdominal muscular response at 0.04-0.06-0.08-0.1 mL distension volumes with 183%, 92%, 39% and 31%, respectively compared to the instillation with UC supernatant incubated only with the vehicle, i.e. DMSO (P<0.001, P<0.05, P<0.05, P<0.01, respectively). This nociceptive response was similar to the hypersensitivity observed after IBS-D supernatant infusion. (Fig. 7A) Incubation of the UC supernatant before infusion with the mixture of serine-protease inhibitors, aprotinin and SBTI, restored the nociceptive response to the level of the healthy subjects supernatant infusion, particularly from 0.02 to 0.08 mL distension volumes (UC vs UC + aprotinin/SBTI, P<0.05) (Fig. 7.B).

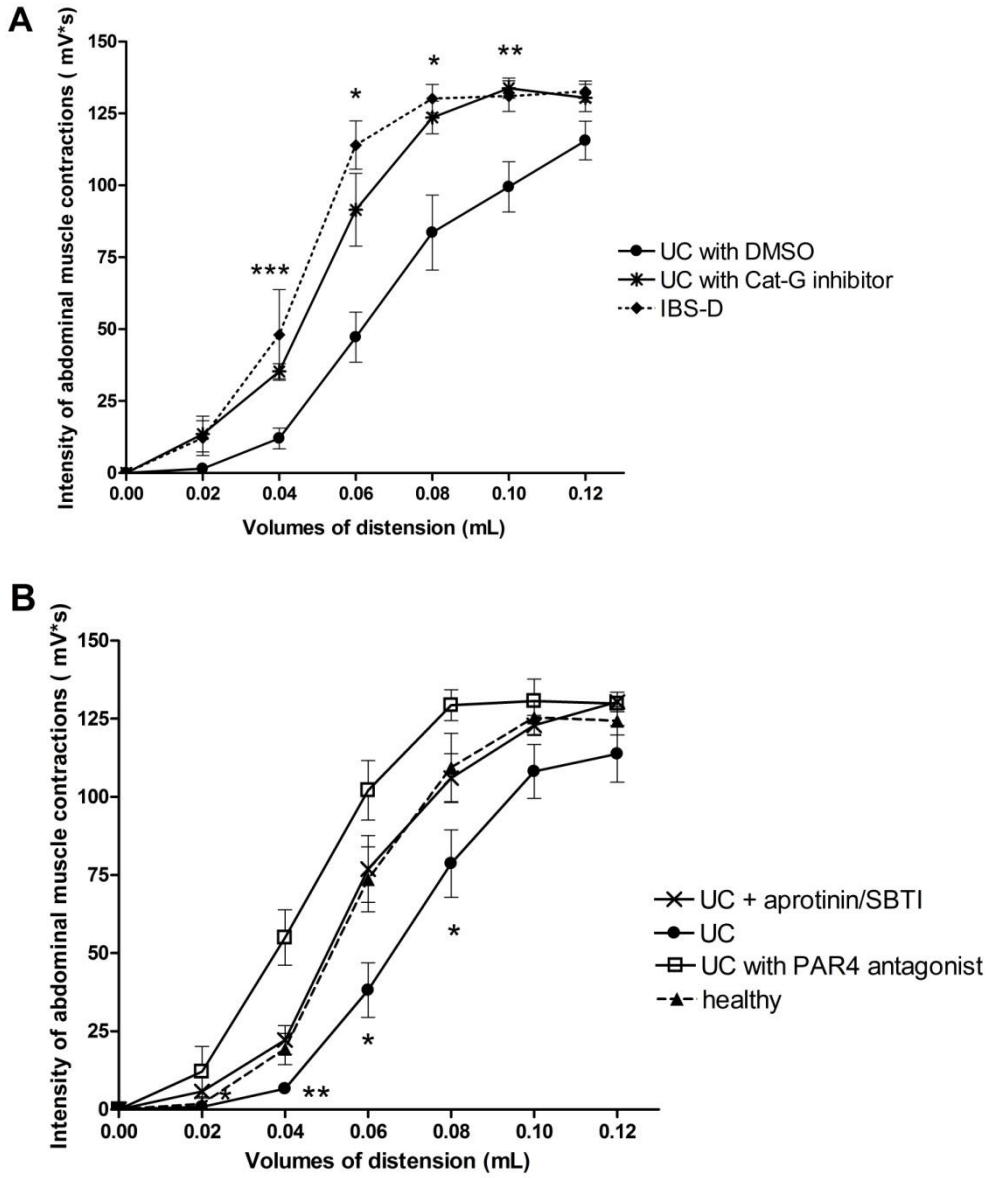


Fig. 7. Effects of inhibition of **A)** cathepsin G or **B)** inhibition of total serine-protease activity in the infused UC supernatant on visceral sensitivity of mice. A) UC with DMSO (n=12) vs UC with Cat-G inhibitor (n=12), *P<0.05, **P<0.01, ***P<0.001. B) UC (n=12) vs. UC with aprotinin/SBTI (n=12), *P<0.05, **P<0.01).

4.2.2.5. Comparative influence of ic. vs ip. administration of PAR-4-AP on colorectal sensitivity to distension

When infused *ic.* prior to distension, PAR-4-AP (100 µg) triggered a hyposensitivity to CRD at the distension volumes from 0.04 to 0.08 mL compared to vehicle infusion ($p<0.05$). In contrast, when injected *ip.* at the same dose, PAR-4-AP had no effect on the response to CRD ($p>0.05$) compared to vehicle. Indeed, abdominal response to CRD after PAR-4-AP administration was significantly different between *ic.* and *ip.* routes at the same distension volumes ($p<0.05$; Fig. 8).

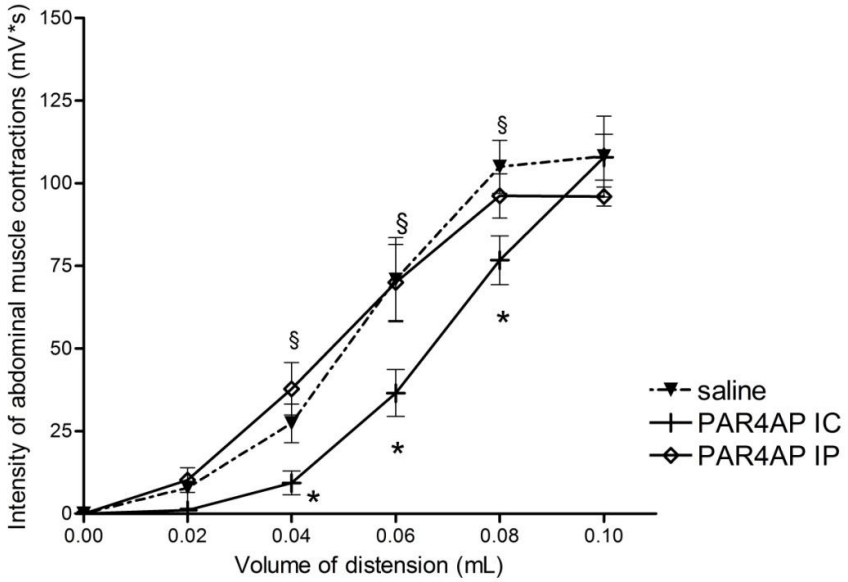


Fig. 8. Effect of PAR-4 activating peptide (PAR-4-AP) on visceral sensitivity by *ip.* administration. *ic.* (n=12) vs. saline (n=8), *$p<0.05$, *ip.* (n=9) vs saline, $p>0.05$. *ic.* vs *ip.* PAR-4-AP, $^{§}p<0.05$.

4.2.2.6. Effects of TAP on PAR-4-AP evoked hyposensitivity

The colorectal infusion of TAP blocked the hyposensitive effect of PAR-4-AP *ic.* infusion (Fig. 9). The PAR-4-AP alone decreased the sensitivity by 54 to 33% at distension volumes of 0.04 to 0.08 mL, respectively ($p<0.05$). To the contrary, PAR-4-AP + TAP did not affect the sensitivity compared to vehicle ($p>0.05$). TAP alone had no effect on colorectal sensitivity ($p>0.05$).

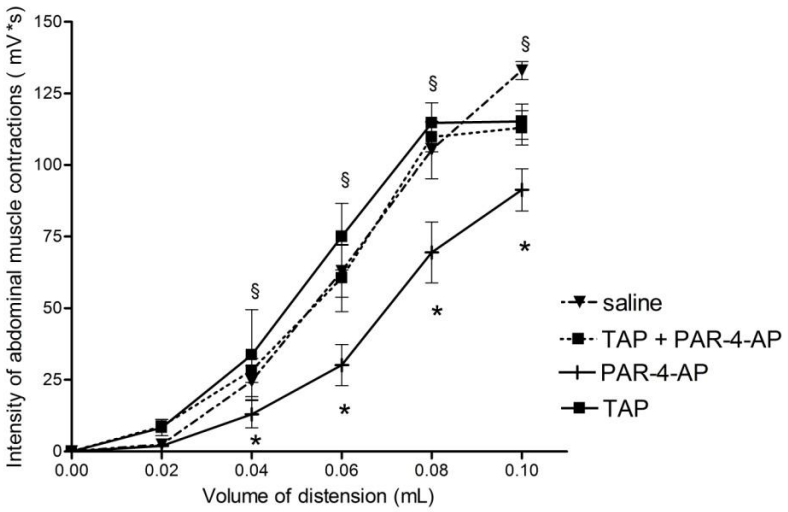


Fig. 9. Effect of PAR-4 activating peptide (PAR-4-AP) in combination with tight junction blockade. PAR-4-AP (n=9) vs PAR-4-AP + TAP (n=12); $^{\S}p<0.05$; while PAR-4-AP vs saline (n=8), *p<0.05. TAP alone (n=8) vs saline, p>0.05.

4.2.2.7. SCID mice

Postoperative complications and mortality was not different in SCID mice compared to their Balb/cBy controls. Compared to their BALB/cBy controls, SCID mice had a significantly greater abdominal response to CRD at the distension levels 0.04 to 0.1 mL increasing the intensity of EMG response by 384% to 132%, respectively (p<0.01; p<0.01; p<0.01; p<0.001). PAR-4 activation effectively reversed this hypersensitivity (p<0.01, p<0.05; p<0.05; p<0.05; Fig. 10).

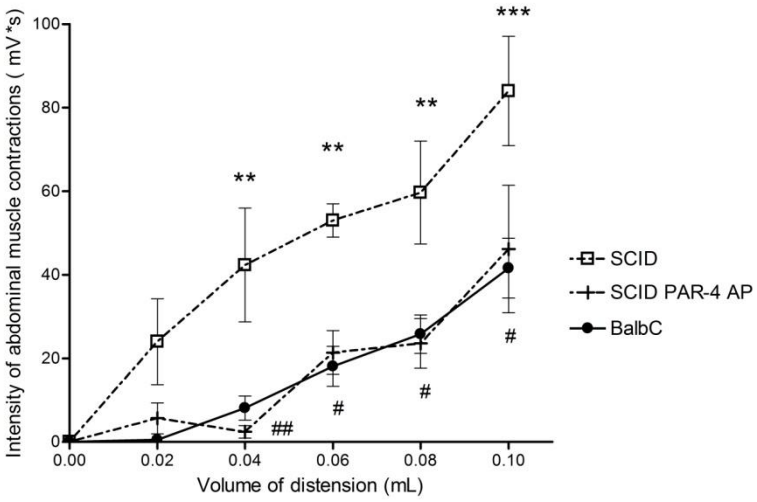


Fig. 10. Effect of PAR-4 activating peptide (PAR-4-AP) on SCID mice**.** SCID mice (n=7) vs BalbC controls (n=8); **p<0.01, ***p<0.001), SCID vs SCID+PAR--AP (n=8) $^{\#}p<0.05$; $^{\#\#}p<0.01$).

4.2.2.8. Effect of PAR-4 activation and blockade in water avoidance stress model

Water avoidance stress (WAS) significantly increased visceral sensitivity to distension by 730%, 119% and 69%, at distension volumes of 0.02, 0.04 and 0.06 mL, respectively ($p<0.01$, $p<0.05$, $p<0.05$, Fig. 11A). *Ic.* infusion of PAR-4 activating peptide (PAR-4-AP) before distension suppressed the colonic hypersensitivity to distension and restored it to the baseline at all distension volumes. Further, the PAR-4 antagonist had no impact on the visceral hypersensitive response to CRD observed in stressed mice (Fig. 11B).

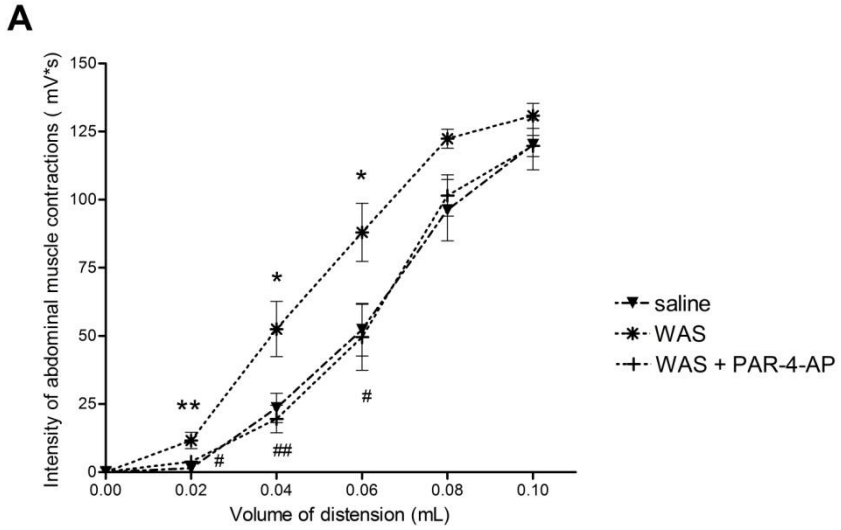


Fig. 11. A) Effect of PAR-4 activation in water avoidance stress model. A) WAS (n=11) vs saline (n=10), *p<0.05, **p<0.01; WAS vs WAS + PAR-4 activating peptide (PAR-4-AP, n=12), $^{\#}p<0.05$, $^{\#\#}p<0.01$.

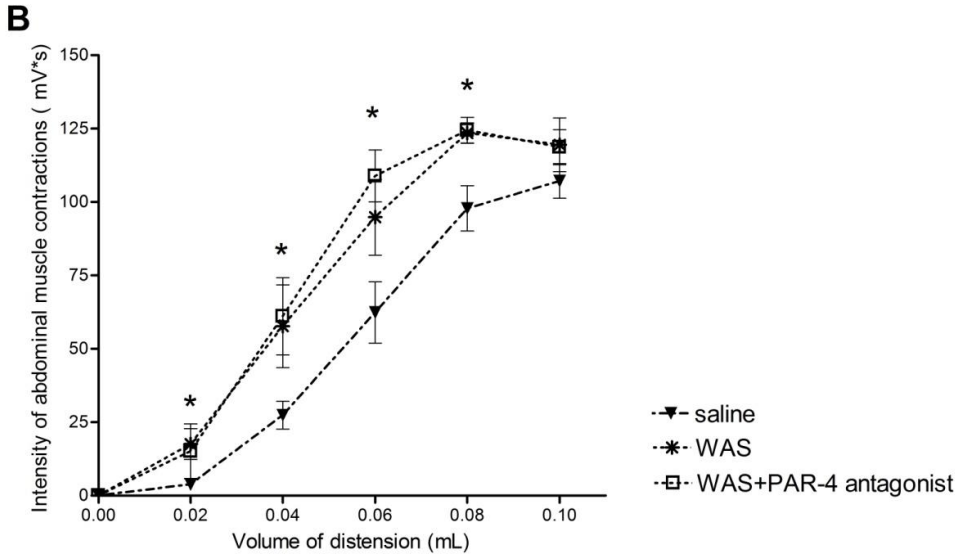


Fig. 11. B) Effect of PAR-4 blockade in water avoidance stress model**.** WAS vs PAR-4 antagonist + WAS (n=10), p>0.05; saline vs WAS, *p<0.05.

4.2.2.9. Effect of PAR-4 activation and blockade in TNBS induced hypersensitivity

72 h after ic. infusion of TNBS, the abdominal response to CRD was enhanced by 1529%, 98% and 90% at distension volumes of 0.02, 0.04 and 0.06 mL, respectively, when compared to controls without TNBS ($p<0.001$, $p<0.05$, $p<0.01$, Fig. 12A). Ic. infusion of PAR-4-AP prior to distension suppressed the increased sensitivity to CRD for all volumes. Unexpectedly, treatment with the PAR-4 antagonist increased the TNBS-induced hypersensitivity by 78%, 42% and 19% at distension volumes of 0.04, 0.06 and 0.08 mL compared to TNBS alone ($p<0.05$, $p<0.05$, $p<0.05$; Fig. 12B).

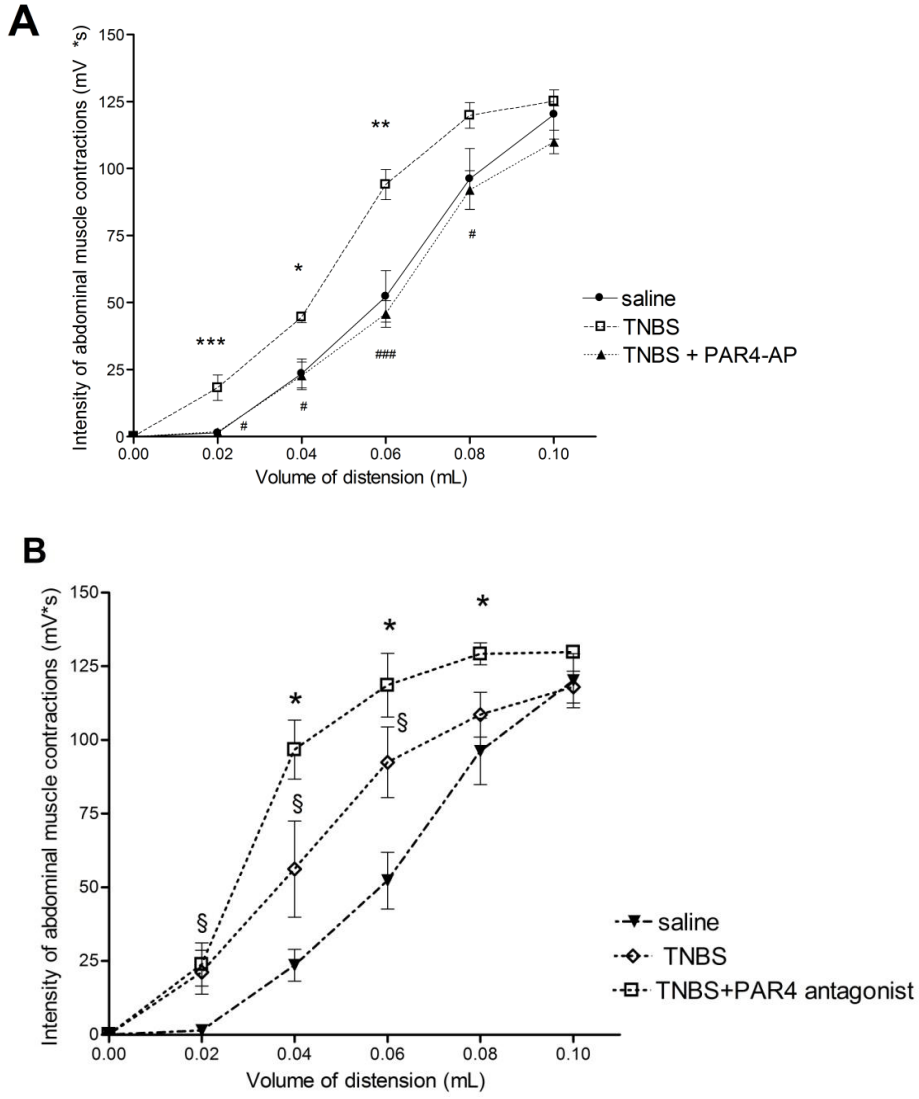


Fig. 12. Effect of PAR-4 activation and blockade in TNBS induced hypersensitivity. **A)** TNBS (n=12) *vs* saline (n=10), *p<0.05, **p<0.01; ***p<0.001. TNBS *vs* TNBS + PAR-4 activating peptide (PAR-4-AP, n=11) $^{\#}p<0.05$, $^{\#\#\#}p<0.001$. **B)** TNBS *vs* TNBS + PAR-4-antagonist (n=9) *p<0.05; saline *vs* TNBS, $^{\S}p<0.05$.

4.2.2.10. Influence of Cat-G on WAS and TNBS induced colorectal hypersensitivity

The *ic.* infusion of Cat-G significantly decreased the hypersensitivity observed in WAS treated mice at the volumes from 0.02 to 0.08 mL ($p<0.05$; $p<0.05$; $p<0.01$, $p<0.01$; Fig. 13A). Surprisingly, Cat-G could not reverse the visceral hypersensitivity induced by low grade TNBS inflammation (saline *vs* TNBS + Cat-G; $p<0.05$ at the distension volumes of 0.04 and 0.06 mL and $p<0.01$ at the volume of 0.08 mL; Fig. 13B).

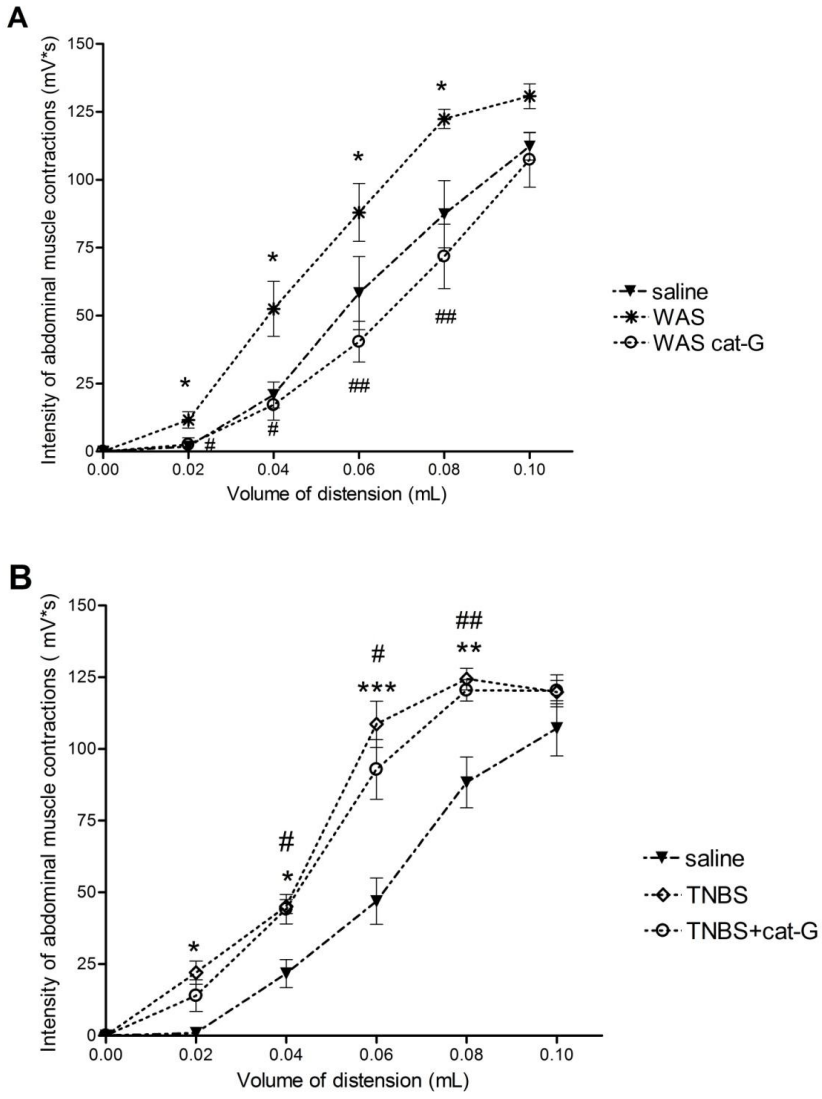


Fig. 13. Effect of Cat-G in water avoidance stress and TNBS induced colorectal hypersensitivity. **A)** saline *vs* WAS, *p<0.05; WAS *vs* WAS + Cat-G, $^{\#}p<0.05$, $^{\#\#}p<0.01$. **B)** saline *vs* TNBS, *p<0.05, **p<0.01, ***p<0.001; saline *vs* TNBS + Cat-G, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

4.2.2.11. Cathepsin-G activity in feces of mice after WAS and TNBS treatment

Cat-G activity was significantly increased in feces of mice with TNBS colitis (17.0 ± 2.6 U/mg prot) compared to naive controls (8.8 ± 1.9 U/mg prot). In contrast Cat-G activity was not elevated in the feces of stressed mice compared to naive controls (6.9 ± 2.5 U/mg prot, $p>0.05$; Fig. 14).

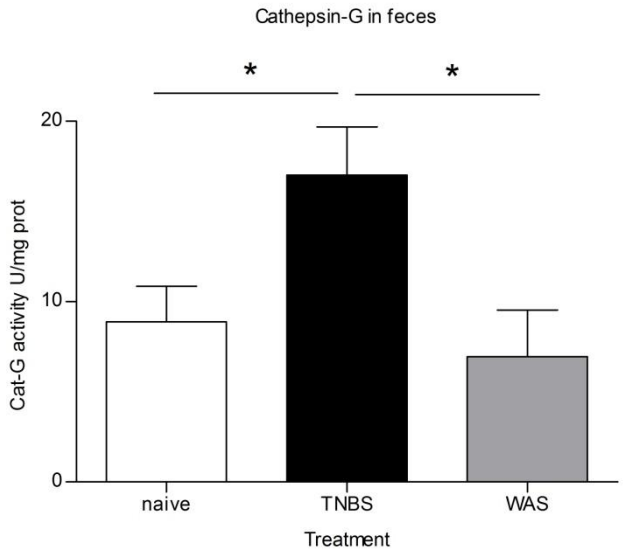
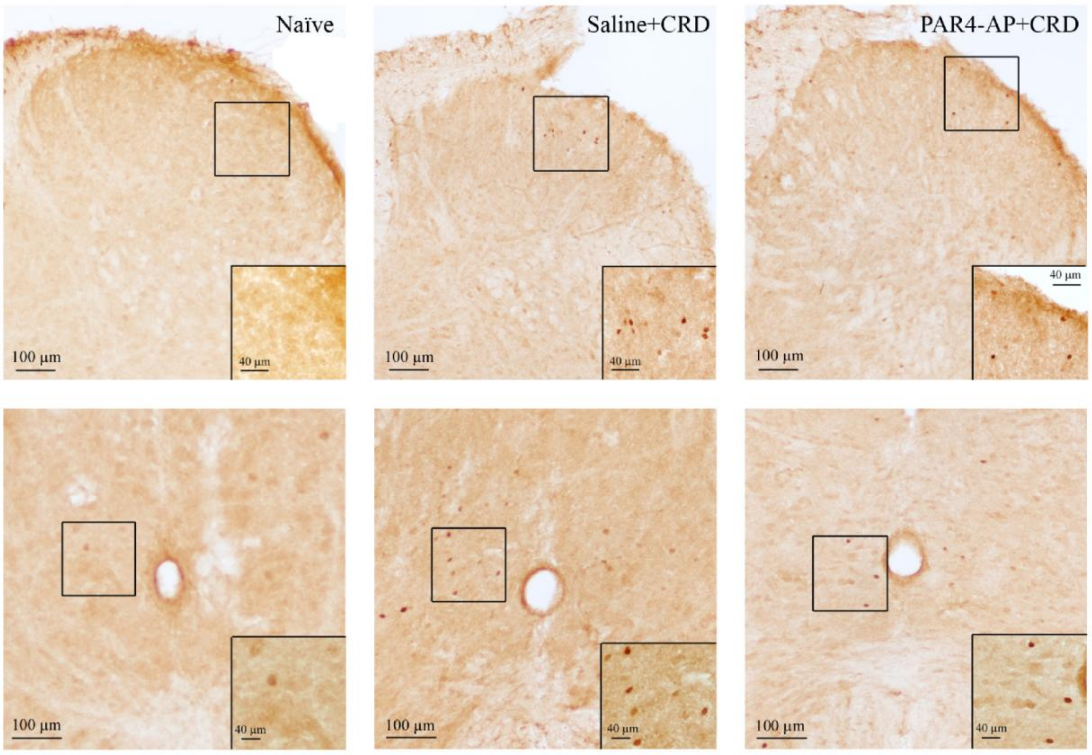


Fig. 14. Cat-G activity in feces of mice 72 h after treatment with TNBS or after 4 WAS sessions. TNBS colitis (n=8) vs naive controls (n=8), *$p<0.05$; TNBS vs WAS (n=10), *$p<0.05$.

4.2.2.12. Effects of PAR-4-AP on the CRD induced spinal Fos expression

In naive mice the number of Fos-positive cells in the L5-S1 levels of the spinal cord was 7.4 ± 0.7 /section. This number was markedly increased (4.9-fold) after CRD in the group treated with saline compared to naive, undistended animals ($p<0.001$; Fig. 15 A, B). However, pretreatment with PAR-4-AP *ic.* infusion reduced the CRD-induced Fos expression increase by 46% in the spinal cord compared with saline ($p<0.001$; Fig. 15 A, B).

A



B

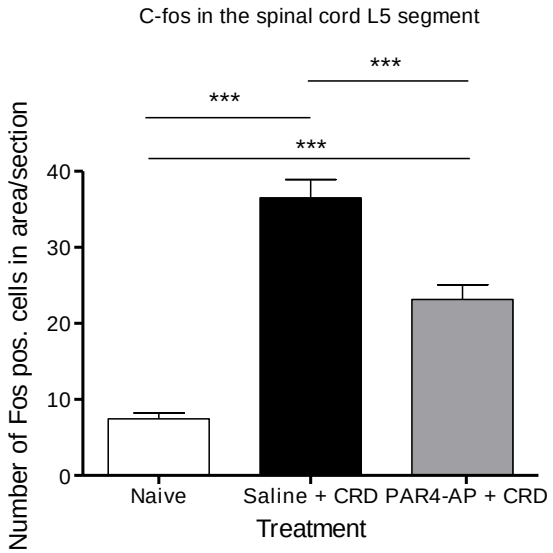


Fig. 15. Effect of PAR-4 activation on CRD induced Fos expression in the spinal cord. **A)** Fos immunostaining of dorsal horn and central area of the spinal cord (segment L5) in naive, undistended animals (n=5), and after CRD in vehicle (n=5) or PAR-4 AP treated (n=5) mice. **B)** Fos expression in the lumbosacral spinal cord after CRD + saline vs naive, ***p<0.001; saline + CRD vs PAR-4-AP + CRD, ***p<0.001.

5. DISCUSSION

The results presented here have confirmed the possible role of cathepsin G and its receptor, PAR-4 in the pathomechanism of ulcerative colitis, and have highlighted their ability to alter visceral pain sensation. The effect of UC fecal supernatants on increasing gut paracellular permeability via Cat-G and PAR-4 activation has been demonstrated previously *in vitro* in Ussing-chambers (29). Our study has confirmed this role of Cat-G in UC fecal supernatants, and the pathway of PAR-4 activation in the permeability increase by an *in vivo* assay on mice, using ^{51}Cr-EDTA. It has been shown recently that the activation of PAR-1 on enterocyte layers increases paracellular permeability (24). This effect is mediated by the direct activation of PAR-1 on the epithelial cells, leading to the initiation of apoptosis at the mucosal surface. PAR-2 activation also induces increased paracellular permeability (19), however, by a different mechanism. A low dose (5 μg) of the PAR-2 agonist (SLIGRL) increases colonic paracellular permeability in mice due to the phosphorilation of MLCK without resulting in inflammation, while a higher dose (100 μg) evokes a marked inflammation and a significantly higher increase in the permeability by an IFγ dependent mechanism (18). Fecal supernatants from IBS-D patients, bearing with elevated serine-protease activity, increased permeability of mice colonic strips *via* PAR-2 activation (35). This study has confirmed the participation of MLCK in PAR-2 signalling, demonstrated by elevated phosphorilated MLC (pMLC) immunolabeling in mice colon and increased level of pMLC shown by Western-blot after the infusion of fecal supernatants from IBS-D patients. Our results show that the permeability increase provoked by Cat-G is also mediated by the activation of MLCK. Namely, the blockade of MLCK with ML-7 erased the effect of Cat-G on paracellular permeability, proving that this effect is mediated through the phosphorilation of MLC. This is in agreement with previous observations on isolated Caco-2 intestinal epithelial cells demonstrating that tight junction opening is driven through MLC phosphorylation, which depends on MLCK activation (87). Our immunohistochemistry results also confirm that *ic.* infusion of fecal supernatants from UC patients, Cat-G and PAR-4 agonist increase the phosphorylation of MLC. Similarly, Western blotting have shown a higher level of pMLC in the colon of mice infused with fecal supernatants from UC patients than those infused with vehicle or supernatants from healthy subjects.

PARs play a role in inflammation. The PAR-4-AP, similarly to the PAR-1 and PAR-2 agonists, stimulates the release of inflammatory mediators in human respiratory epithelial cell lines (8). Further, it generates oedema and granulocyte recruitement by means of the components of the kallikrein-kinin system when injected into the mice hind paw, which can be reversed by the PAR-4-antagonist (46). Furthermore, PAR-2 activation has been shown to trigger colitis (19, 90). PAR-2

agonist induced granulocyte recruitement, wall thickening, tissue damage, and elevated T-helper cell type 1 cytokine levels in the colon of mice, which effects were not seen in the PAR-$2^{-/-}$ knock outs (19). The same role of PAR-4 and PAR-3 in colonic inflammation has never been investigated. The activity of MPO, an enzyme of the neutrophil granulocytes, correlates with neutrophil infiltration in the colon, therefore the assessment of MPO activity is widely used for quantification of intestinal inflammation (68). Hence we have chosen this method to examine the effect of UC supernatants on colonic inflammation in mice. IC infusion of serine-protease-rich supernatants from UC patients increased MPO activity already after four hours from the beginning of administration. Preincubation of UC fecal supernatants with Cat-G inhibitor abolished the MPO increase, demonstrating that Cat-G activity in the feces is able to trigger inflammation, resulting in the accumulation of neutrophils. The MPO increase evoked by UC supernatants was mimicked by the administration of PAR-4-AP, and blocked by the PAR-4-antagonist, suggesting that PAR-4 is mediating the effect. Additionally, ML-7 also blocked the MPO elevation provoked by Cat-G, which proves that the invasion of neutrophils is linked to the permeability increase. Previously it has been shown that the blockade of MLC phosphorylation by ML-7 prevented the colonic paracellular permeability increase and the consequent bacterial translocation and colonic inflammation after lipopolysaccharide administration in rats (66). Thus, Cat-G present in UC fecal supernatants is able to trigger an inflammatory reaction via PAR-4 activation, likely through colonic epithelial barrier disruption.

Previous studies have shown elevated protease activity in fecal supernatants from UC patients similarly to IBS-D patients (29, 35). This high protease activity in fecal supernatant of IBS-D patients triggered visceral hypersensitivity in mice by activating PAR-2 (35). Nevetheless, we have shown that fecal supernatants from UC patients induce hyposensitivity to colorectal distension when infused *ic.* in mice, contrary to the hypersensitivity found in the same experimental conditions with fecal supernatant from IBS-D patients. By neutralizing serine-proteases in fecal supernatants from UC patients with aprotinin and SBTI, colorectal hyposensitivity is reversed, suggesting that the antinociceptive effect of these supernatants is linked to serine-proteases. These results clearly demonstrate a PAR mediated modulation of visceral pain by serine-proteases contained in feces from IBS-D and UC patients. While the IBS-D fecal supernatant evoked colorectal hypersensitivity was mediated by PAR-2 activation, the UC supernatants provoke a hyposensitivity *via* PAR-4 activation, as the hyposensitive effect of UC supernatants is blocked by the PAR-4-antagonist, and the hyposensitivity is reproduced by PAR-4-AP. Further, Cat-G activity was not elevated in the feces of IBS-D patients, contrary to that of UC patients, but when Cat-G was added to the IBS-D supernatant, the hypersensitive effect of the latter was reversed. Cat-G inhibitor blocks the hyposensitive effect of UC supernatants, while Cat-G infusion also causes a hyposensitivity in mice. These results make

Cat-G an ideal candidate responsible for the hyposensitivity evoked by UC supernatants in mice though PAR-4 activation.

The combination of the PAR-4 antagonist or the Cat-G inhibitor with the perfusion of UC fecal supernatant led us to interesting observations. The blockade of PAR-4 or Cat-G not only erased the hyposensitivity to distension, but evoked a colorectal hypersensitivity. These results suggest that serine-proteases, which are contained in fecal IBS-D supernatant and activate PAR-2 to evoke PAR-2 mediated hypersensitivity are also present in fecal supernatant from UC patients but are unable to counterbalance the antinociceptive effects of Cat-G. Thus the opposite effects of IBS-D and UC supernatants on visceral sensitivity in mice are related to the difference in the type and constitution of serine-proteases between fecal supernatants of IBS-D and UC patients. Stools from healthy subjects contain a certain, limited quantity of serine-proteases originating from different sources, such as digestive enzymes, inflammatory cells and microflora (77). According to our hypothesis, they create an equilibrium of basal visceral sensitivity by activating equally PAR-2 and PAR-4 (Fig. 16). Fecal supernatants from IBS-D and UC patients both contain a high amount of serine-proteases compared to healthy subjects, which is not accompanied by elevated fecal mast cell tryptase and pancreatic elastase-1 (77), excluding a pancreatic or mast cell origin. 15% of serine-protease activity in UC fecal supernatants is linked to Cat-G (29). However, elevated serine-protease activity in fecal supernatant from IBS-D patients is related to other serine-proteases than Cat-G, possibly originating from the microflora (77), evoking visceral hypersensitivity by PAR-2 activation. PAR-4 does not play a role in this process. To the contrary, serine-proteases in fecal supernatants of UC patients are able to activate both PAR-2 and 4, but they induce predominantly a PAR-4 activation due to their Cat-G content, consequently they provoke visceral hyposensitivity. (Fig. 16).

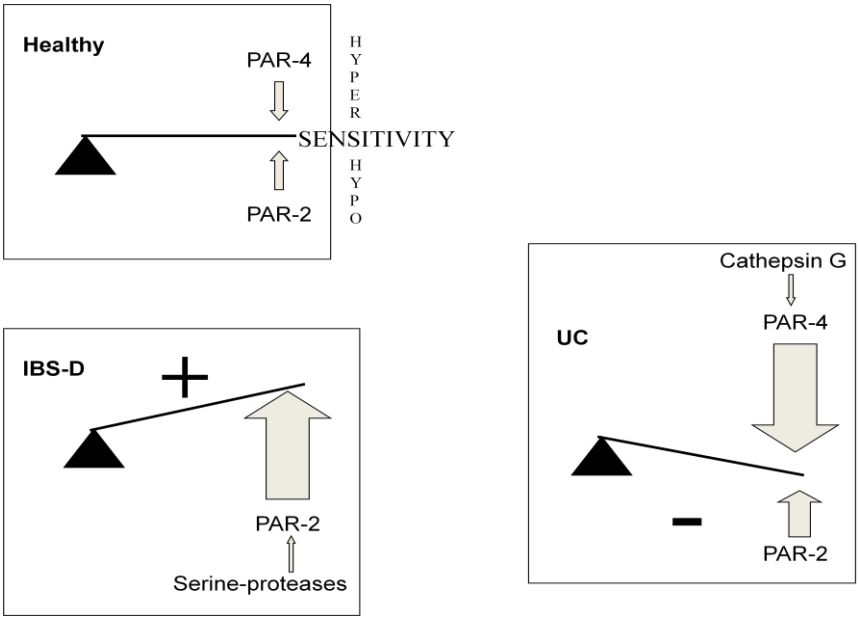


Fig. 16. Schematic representation of visceral sensitivity based on the balance between the activation of PAR-2 and PAR-4. In healthy subjects, there is a basal fecal serine protease activity which may allow the low activation of both PAR-2 and 4, but hyper- and hyposensitive effects are balanced, creating an equilibrium. However, in IBS-D, PAR-2 is stimulated by the elevated fecal serine-protease activity, resulting in visceral hypersensitivity. To the contrary, PAR-4 is overactivated by cathepsin G contained in UC supernatant, causing a hyposensitivity

Nevertheless, serine-proteases different from Cat-G in UC supernatant are able to activate PAR-2. This effect merges to the surface after the blockade of PAR-4 or Cat-G by a PAR-4 antagonist and a Cat-G inhibitor, respectively, when hypersensitivity - similar to that obtained with IBS-D supernatant infusion - appears (Fig. 17). Furthermore, Cat-G added to the infused IBS-D supernatant inhibits the pronociceptive effect of the IBS-D supernatant (Fig. 17). All these observations support our hypothesis that the hypersensitivity caused by fecal supernatants from UC patients combined with PAR-4 antagonist or Cat-G inhibitor is linked to serine-proteases activating PAR-2.

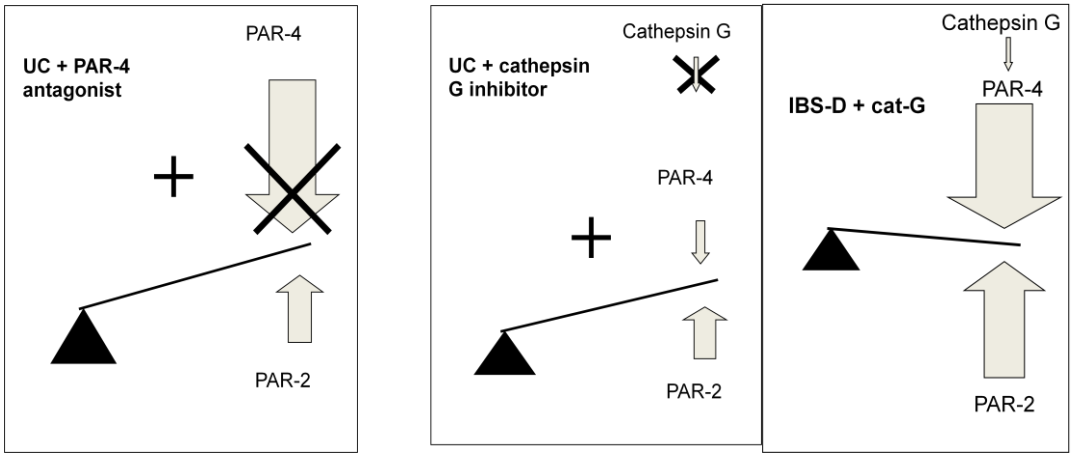


Fig. 17. The presence of PAR-2 activators in the UC supernatant merges to surface when PAR-4 or cathepsin G is blocked, and consequently visceral hypersensitivity appears. When cathepsin G is associated to the infused IBS-D fecal supernatant, the hypersensitive effect of IBS-D supernatant is reversed.

Apart from colonic epithelium (67), PAR-4 has been found on dorsal root ganglion (DRG) neurons (7) and different immune cells such as Kupffer cells, B-lymphocytes (78) and mast cells (63). In the next part of our work we aimed to localize the receptors responsible for the observed visceral hyposensitivity. First, our results showed that systemic (*ip.*) administration of PAR-4-AP did not reduce pain sensation to CRD. This finding suggests that the site of action is not central, but probably peripheral, similarly to previous observations, when intraplantar injection of PAR-4-AP was antinociceptive in the injected paw, but not in the contralateral paw (7). Moreover, TAP, a tight junction blocker suppresses the antinociceptive effect of PAR-4-AP, suggesting that the responsible receptors are located beyond the epithelial cell layer. TAP is a tight junction blocker used for decades to block cation-selective permeability in various epithelial tissues such as gallbladder, small intestine or choroid plexus (64, 65). In mouse and rat colon, TAP blocked the permeability increase provoked by PAR-2 activation (20) or acut stress (1). In the latter, a link has been established between colonic permeability and visceral hypersensitivity (1), when the blockade of permeability increase by a myosin-light chain blocker, ML-7 or TAP erased the augmentation of visceral sensitivity evoked by acut stress. Nevertheless, in our case the PAR-4 elicited increase in colonic permeability is associated with a decrease of colorectal sensitivity, which may seem controversial. According to our explanation, increase of permeability does not rule out the antinociceptive effect, to the contrary, the elevated permeability may facilitate the PAR-4 agonist to reach the nerve terminals, which seems essential in the mechanism.

As a next step we have shown that colonic lymphocytes do not play a role in the antinociceptive effect of PAR-4-AP. SCID mice, which bear with functionally defective T and B

cells due to a mutation in the Prkdc gene, were discovered in 1983 (14). Since they have been widely used in the research of immunological diseases, transplantation and cancer, and lately they were found hyperalgesic to CRD (91). Reconstitution of the mice with CD4+ T cells restored visceral pain sensation within the normal limits (91). However, the antinociceptive effect of PAR-4-AP is still present in SCID mice suggesting that such action does not require functional B or T cells. Nevertheless, the involvement of other immune cells, like macrophages cannot be excluded with the use of SCID mice, as PAR-4 has been described also on the macrophages of rats (49).

Induction of Fos, the immediate-early protooncogene expression is a marker of neuronal activation (37). CRD activates primary visceral sensory afferents and induces Fos expression bilaterally in the L6-S2 spinal segments with no difference between the sides in rats (86). In mice, colonic primary sensory afferents project to the lumbosacral spinal cord (L5-S1) particularly in laminae I and II located in the dorsal horn (25). Recently it has been reported that a probiotic treatment with Lactobacillus farciminis, which decreases the stress induced colorectal hypersensitivity in rats, attenuates the overexpresson of Fos protein in the spinal cord after CRD in stressed animals (2). Our data confirmed a Fos protein overexpression in mice after CRD in the L5-S1 segments of the spinal cord. Further, parallel to the decreased sensitivity to CRD, PAR-4-AP attenuates Fos expression in the lumbosacral spinal cord, suggesting a moderated activation of the primary sensory neurons. It is in accordance with previous results shown by patch clamp recordings that PAR-4 activation in DRG neurons projecting to the colon suppresses their excitability (50). However we cannot exclude that PAR-4-AP may activate receptors located on vagal afferents, as they were shown to control nociception and to modulate mechanical hyperalgesic behaviour (47), and reduce formalin-induced Fos-expression in the nucleus caudalis in rats (13). Recently it has been reported by in situ hibridization that PAR-4 is broadly distributed on all neuronal sizes. (89) Based on their functional studies, the authors conclude that the activation of PAR-4 in large-diameter myelinated afferents may have an analgesic action, while in small-diameter nociceptive afferents they can potentiate TRPV1 and enhance the release of neuropeptides (89) This theory may provide explanation for the inflammatory effects of the PAR-4 agonists parallel to its antinociceptive effects.

Water avoidance stress and TNBS induced colitis are two well described models of colonic hypersensitivity. Stress is followed by the activation of colonic mast cells (38) resulting in a colonic barrier dysfunction (81), which leads to an enhanced antigene uptake already after 2 h (52). Mast cell degranulation may provoke permeability changes and hypersensitivity in the colon by the activation of PAR-2 due to the released tryptase (30). In contrast in colitis, a vaste amount of inflammatory mediators are released to the colonic wall, such as bradikinin (27) or prostaglandins (36), which can trigger higher response to colonic distension. Inflammatory mediators may sensitize colonic afferents

to enhance firing in response to painful stimuli (12). A study on rat spinal neurons showed a significantly increased activity to CRD in acute colonic inflammation (71). Thus, the pathomechanism of hypersensitivity may differ in these two models, but our results demonstrate that PAR-4-AP exerts similar antihyperalgesic effects. However, by testing the PAR-4 antagonist in our two hypersensitivity models, we have obtained different results. Surprisingly, in the TNBS colitis model the colorectal hyperalgesia was augmented by blocking PAR-4 with an antagonist, while there was no change in visceral sensitivity in the stress model. This suggests the presence of an endogenous activation of PAR-4 in colonic inflammation, exerting a feed-back antinociceptive effect. One explanation may be that in TNBS colitis a massive invasion of neutrophil granulocytes appears in the colonic mucosa, acting as a source of inflammatory mediators, such as Cat-G, a potent activator of PAR-4 (79) that reach sensitive nerve terminals and cause hyposensitivity to CRD. In agreement with such hypothesis, we found an increased Cat-G activity in the feces of mice with TNBS colitis, but not in that of stressed mice. This may highlight Cat-G in a new role as an endogenous PAR-4 agonist activating a feed-back loop to decrease pain in inflammation.

Intriguingly, the *ic*. administration of Cat-G reversed the colorectal hypersensitivity in mice evoked by WAS, but not by TNBS, contrary to PAR-4-AP which was effective in both models. This finding can be explained by the non-specificity of Cat-G on PAR-4, that is, Cat-G may disarm PAR-1 and PAR-2 on human bronchial fibroblasts (41), and it induces apoptosis via a PAR-independent mechanism in cardiomyocytes (37). On the other hand, PAR expression may be influenced by inflammatory mediators released in certain conditions, therefore local PAR signalling may be altered (41, 42). For instance, TNF-α and LPS upregulates PAR-2 and induces PAR-4 mRNA expression in human bronchial fibroblasts (41). Likewise, PAR expression may also be altered in TNBS induced colonic inflammation, when Cat-G acts differently on PARs due to its non-specificity. To our explanation, in the WAS model, Cat-G release to the colonic wall is not elevated, and endogenous PAR-4 activation is not significantly present, therefore extrinsic Cat-G exerts an analgesic effect by PAR-4 activation, similarly to PAR-4-AP. However, in the TNBS model, endogenous Cat-G is already highly present in the colonic wall, and provokes analgesia *via* PAR-4. While PAR-4-AP, a pure PAR-4 agonist is still antinociceptive in this model, additional extrinsic Cat-G does not reinforce the analgesic effect, probably due to its non-specificity.

Taken together, we have identified a new factor, fecal Cat-G in UC patients that may play an important role in the pathogenesis of the disease by increasing colonic epithelial permeability and inducing mucosal inflammation via PAR-4 activation. Further, by direct activation of PARs located on colonic nerve terminals, Cat-G is a possible candidate to activate an endogenous antinociceptive mechanism in colitis.

6. NEW RESULTS ESTABLISHED IN THE THESIS

1. UC fecal supernatants increase colonic paracellular permeability *in vivo* infused intracolonically (*ic.*) in mice, and this increase in colonic paracellular permeability is related to PAR-4 activation and the presence of Cat-G in the fecal supernatants.
2. UC fecal supernatants can be responsible for triggering a colonic inflammation, as early as 4 h after the beginning of administration, and this inflammation is linked to the presence of Cat-G and the activation of PAR-4. This colonic inflammation is connected to the increase of colonic paracellular permeability.
3. UC fecal supernatants rich in Cat-G can trigger visceral hyposensitivity in mice due to the activation of PAR-4.
4. The visceral hypersensitivity triggered by *ic.* infusion of fecal supernatants from IBS-D patients is not PAR-4 mediated, and it can be reversed by PAR-4 activation with Cat-G.
5. The colorectal antinociceptive effect of PAR-4 activating peptide (PAR-4-AP) is driven locally and is not dependent on lymphocytes.
6. The intracolonic activation of PAR-4 by PAR-4-AP has antinociceptive effect in two different visceral hypersensitivity models, water avoidance stress model and TNBS induced colitis.
7. The thesis provides new information on the presence of an endogenous activation of PAR-4 in inflammatory mediated hypersensitivity acting as a feed-back mechanism controlling pain, not activated in stress-induced visceral hyperalgesia.

7. SUMMARY

BACKGROUND: A growing body of evidence demonstrates that besides genetic predisposition, luminal factors participate to trigger colonic inflammation in inflammatory bowel diseases and particularly in ulcerative colitis (UC). Tight junction alterations are present in the colonic epithelium in active Crohn's disease, leading to epithelial barrier dysfunction. Further, increased intestinal permeability can predict relapse in inactive Crohn's disease patients. This increased permeability may facilitate the penetration of luminal components, including bacteria, into the mucosa, and an abnormal immune response to these products can lead to neutrophil infiltration and cytokine-driven inflammation.

The acute phase of UC is characterized by colonic mucosal injury and the presence of leukocytes including large numbers of neutrophils in the area of tissue damage. Cathepsin-G (Cat-G) is a serine protease that represents approximately 20% of the neutrophil azurophilic granule proteins,

and is capable of binding and activation of proteinase activated receptor (PAR)-4. PARs, a family of 7-transmembrane G-protein-coupled receptors which are activated by the cleavage of their N-terminal domain by serine-proteases, play an important role in gastrointestinal physiology and pathophysiology. PAR-2 activation caused elevated colonic permeability in rats, and Cat-G rich fecal supernatants from UC patients increased permeability of mice colon *in vitro* and provoked gut inflammation *in vivo* by PAR-4 activation. Furthermore, PARs are involved in the sensation of visceral pain. Intracolonic *(ic.)* infusion of the PAR-2 agonist has been shown to induce colorectal hypersensitivity to distension in rats, and PAR-2 activation by high level of serine-proteases may be responsible for the pathogenesis of diarrhea-predominant irritable bowel syndrome (IBS-D).

AIMS: to compare the activity of Cat-G and protease activity in the feces of UC and IBS-D patients to that of controls, to measure if fecal supernatants from UC patients change colonic paracellular permeability *in vivo*, and if this effect is reproducible by PAR-4-AP and Cat-G, to test if fecal supernatants from UC patients, PAR-4-AP or Cat-G are able to provoke colonic inflammation, to explore if the effects on colonic paracellular permeability and inflammation are linked to myosin light chain kinase (MLCK) activation, to evaluate if fecal supernatants from UC patients affect colorectal mechanical sensitivity, and if this effect is reproducible by PAR-4-AP and Cat-G, to localize the receptors participating in the effect on visceral sensitivity, to test the effect of PAR-4-AP in two visceral hypersensitivity models: inflammatory (2,4,6-trinitrobenzene sulfonic acid, TNBS) and non-inflammatory (water avoidance stress, WAS) models.

MATERIAL AND METHODS: Fecal samples from collected from healthy subjects, IBS-D and UC patients and fecal supernatants were prepared. Total serine protease activity and Cat-G activity were measured in fecal supernatants. UC fecal supernatants, pretreated or not with specific Cat-G inhibitor (Cat-G inhibitor), PAR-4 activating peptide (PAR-4-AP, AYPGKF-NH_2) or Cat-G were infused in mice and MPO activity and colonic paracellular permeability were determined. The possible involvement of myosin light chain kinase (MLCK) activation was tested by ML-7 (2 mg/kg intraperitoneally, *ip.*), a specific blocker of MLCK. Colonic paracellular permeability was measured by ^{51}Cr-EDTA. EMG response to colorectal balloon distension (CRD) was recorded in mice following intracolonic *(ic.)* infusion of fecal supernatants from healthy subjects, IBS-D and UC patients or PAR-4-AP or Cat-G. This nociceptive response was also determined after treatment with pepducin (PAR-4 antagonist, P4pal-10) on UC supernatant or after a preincubation with antiproteases or Cat-G inhibitor. Further, mice received PAR-4-AP or vehicle *ip.* or PAR-4-AP *ic.* with or without 2,4,6-triaminopyrimidine (TAP), a chemical tight junction blocker, before CRD. SCID mice were used to test the role of lymphocytes in the antihyperalgesic effect. The effects of

PAR-4-AP and PAR-4-antagonist were evaluated in WAS model and mild acute TNBS colitis. Spinal Fos protein expression was visualized by immunohistochemistry.

RESULTS: Fecal serine protease activity was elevated in samples from IBS-D and UC patients compared to controls. Cat-G levels were elevated in fecal samples from UC patients, but not in those from IBS-D patients. UC fecal supernatants, Cat-G and PAR-4 agonist increased both colonic paracellular permeability and MPO activity. Moreover, the increase of paracellular permeability triggered by Cat-G was blocked by 92.3% by ML-7. Further, the enhanced MPO activity produced by Cat-G infusion was decreased by ML-7 by 43.8 %. In contrast to IBS-D supernatant, UC supernatant promoted colonic hyposensitivity to distension, an effect mimicked by PAR-4-AP or Cat-G. UC supernatant-induced hypoalgesia was inhibited by a cocktail of antiproteases. However, blockade of PAR-4 or Cat-G inhibition resulted in colonic hypersensitivity similar to that observed after IBS-D supernatant infusion. The combination of Cat-G with IBS-D supernatants reversed its nociceptive effect. The antinociceptive effect of PAR-4-AP disappeared when administrered *ip.*, or with the blockade of colonic epithelial tight junctions, suggesting that PAR-4-AP needs to reach directly the nerve terminals in the colon. CRD induced spinal Fos overexpression was reduced by 43% by PAR-4-AP. PAR-4-AP was antihyperalgesic in both hyperalgesia models and in mice defective to lymphocytes. PAR-4-antagonist significantly increased the TNBS, but not the WAS induced colonic hyperalgesia.

CONCLUSIONS: These observations suggest that luminal factors such as PAR-4 and Cat-G play an important proinflammatory role in the pathogenesis of colitis. Moreover, we can conclude that this role is mainly depending on increasing colonic paracellular permeability. Despite similarly elevated serine-protease activities, IBS-D and UC fecal supernatant display visceral pro- and antinociceptive effects in mice, respectively. Visceral hyposensitivity induced by fecal supernatant from UC patients results from PAR-4 activation by cathepsin-G, counterbalancing the pronociceptive effect of simultaneous PAR-2 activation. The antinociceptive effect of PAR-4-AP depends upon its penetration to the colonic mucosa. PAR-4 activation is endogenously involved as a feedback loop to attenuate inflammatory colonic hyperalgesia to CRD.

8. ABBREVIATIONS

ANOVA: analysis of variance, **CAI:** clinical activity index, **Cat-G:** cathepsin G, **CD:** Crohn's disease, **CRD:** colorectal distension, **^{51}Cr-EDTA:** ^{51}Cr-ethylenediamine tetra-acetic acid, **DAPI:** 4',6-diamidino-2-phenylindole, **DMSO:** dimethyl-sulphoxide, **DRG:** dorsal root ganglion, **EMG:** electromyographic, **FITC:** fluorescein isothiocyanate, **HEPES:** 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid, **HTAB:** hexadecyl trimethylammonium bromide buffer, **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase, **IBD:** inflammatory bowel diseases, **IBS:** irritable bowel syndrome, **IBS-C:** irritable bowel syndrome with constipation, **IBS-D:** irritable bowel syndrome with diarrhea**, IBS-M:** irritable bowel syndrome with mixed stool pattern, **IBS-U:** unclassified irritable bowel syndrome, ***ic.*:** intracolonically, ***ip.*:** intraperitoneally, **MLC:** myosin light chain, **MLCK:** myosin light chain kinase, **MPO:** myeloperoxidase, **PA:** protease activity, **PAR-AP:** PAR-activating peptide, **PAR:** proteinase-activated receptor, **PBS:** phosphate-buffered saline, **pMLC:** phosphorylated MLC, **SBTI:** soy bean trypsin inhibitor, **SCID:** severe combined immunodeficient, **SDS:** sodium dodecyl sulfate, **SEM:** standard error of the mean, **TAP:** 2,4,6-triaminopyrimidine**, TCA:** trichloracetic acid, **TNBS:** trinitrobenzene sulfonic acid, **TRPV-4:** transient receptor potential vanilloid receptor-4, **UC:** ulcerative colitis, **UN:** unit, **WAS:** water avoidance stress.

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11. ANNEXES

Annex I.

Annex II.

Annex III.